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# Desmids from the Sinsiru Island in the Middle Kuriles.

Yoshikazu OKADA.

(With 6 Plates and 1 Text-figure)

*Received February 25, 1938.*

## Introduction

Although the study of Desmids in the Middle Kuriles is of great scientific interest, no one has yet attempted to investigate them. This is probably due to the difficulties of traveling there. There is neither inn nor shop in this district, and even roads have never been constructed.

The general public is prohibited to land without the special permission of the authorities, because of the protection or the propagation of precious fur-animals of the land and sea.

Fortunately, in the summer of 1936, I was permitted for 20 days to land on two main islands of the Middle Kuriles, the Sinsiru and Uruppu Islands, for the purpose of the algal investigation.

During my three days' sojourn in the Sinsiru Island I collected the material from the northern part of the island only, since in the southern, as well as middle part, it seemed to be extremely poor because of scarcity of the interior waters.

The materials were secured at the following stations on the respective dates:—

STATION I. July 20, 1936. Eastern foot of Mt. Mikazuki; about 3 pools are found there and the largest one is measured ca. 55 × 27 meters in diameter.

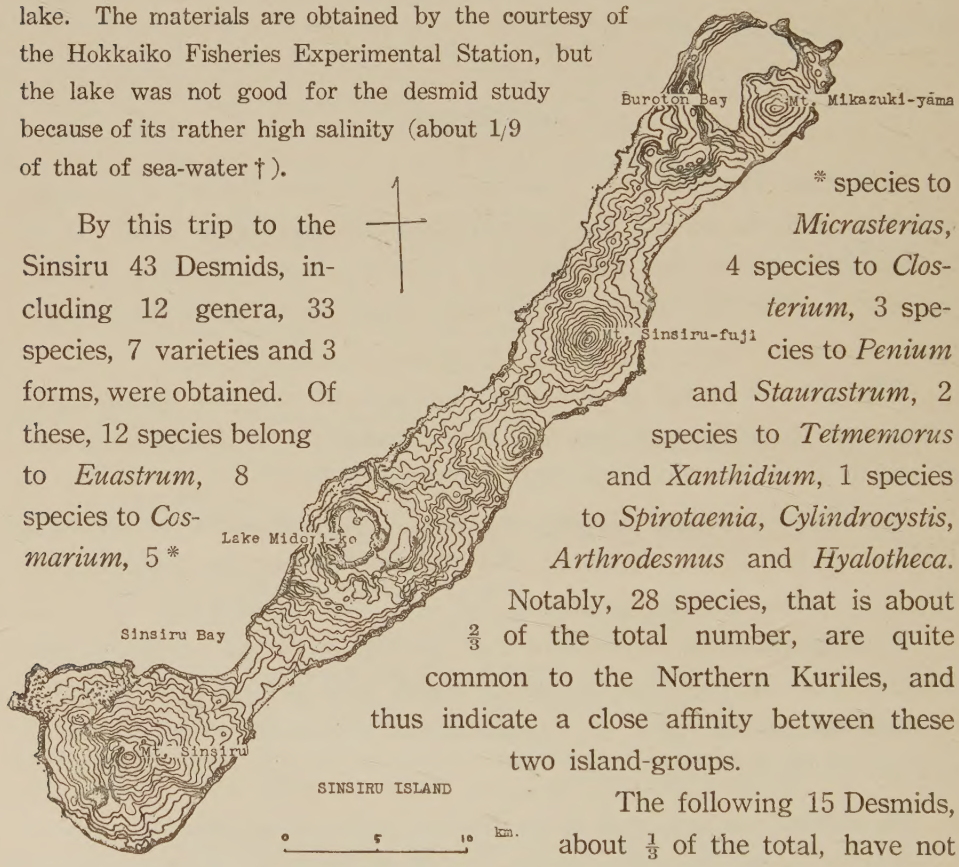
STATION II. July 21, 1936. South-estern foot of Mt. Mikazuki; about 5 pools

The expense of this work was defrayed by the research fund from the Hattori Gratitude Foundation (*Hattori Hôkôkwai*).

are scattered in the dampy plateau (ca. 100 m. above the sea-level) (*cf.* Pl. 6) and most of the pools are very shallow. Water Temp. 19 C°, Air Temp. 13 C°.

STATION III. July, 20, 1936. A small pool near the shore of the Buroton Bay in the north-eastern part of Mt. Mikazuki. None of the desmid was examined and many diatoms (*Surillera* and *Melosira* etc.) and a few sterile fragments of *Spirogyra* are found.

STATION IV. Aug., 1936. Lake Midoriko (meaning "green lake"), the crater lake. The materials are obtained by the courtesy of the Hokkaiko Fisheries Experimental Station, but the lake was not good for the desmid study because of its rather high salinity (about 1/9 of that of sea-water †).




By this trip to the Sinsiru 43 Desmids, including 12 genera, 33 species, 7 varieties and 3 forms, were obtained. Of these, 12 species belong to *Euastrum*, 8 species to *Cosmarium*, 5 * species to *Micrasterias*, 4 species to *Closterium*, 3 species to *Penium* and *Staurastrum*, 2 species to *Tetmemorus* and *Xanthidium*, 1 species to *Spirotaenia*, *Cylindrocystis*, *Arthrodesmus* and *Hyalotheca*. Notably, 28 species, that is about $\frac{2}{3}$ of the total number, are quite common to the Northern Kuriles, and thus indicate a close affinity between these two island-groups.

The following 15 Desmids, about $\frac{1}{3}$ of the total, have not yet been reported from the Northern Kuriles hitherto.

*Cylindrocystis crassa* DE BARY; *Penium didymocarpum* LUND.; *Penium spiros-*

† HARADA, J.: New Cent. Cone in Midoriko Crater Lake. Bull. Volcano. Soc. Jap. Vol. 2, no. 1, p. 96. 1934.

*permum* JOSH.; *Closterium striolatum* EHRENB.; **Euastrum ansatum* RALFS var. *pyxidatum* DELP.; *Euastrum binale* (TURP.) EHRENB.; *Euastrum binale* (TURP.) EHRENB. form. *hians* WEST; **Euastrum verrucosum* EHRENB. var. *alatum* WOLLE; **Micrasterias conferta* LUND.; **Micrasterias truncata* (CORDA) BRÉB. var. *turgida* TAYLOR; **Cosmarium dentiferum* CORDA; **Cosmarium ornatum* RALFS; **Cosmarium praemosum* BRÉB.; **Staurastrum nodosum* W. & G. S. WEST; **Staurastrum orbiculare* RALFS.

Of these Desmids, 14 species, excepting *Closterium striolatum* EHRENB., which is known in Hokkaido already, are newly recognized as Japanese species and 8 species, marked each with an *, are new to Asia.

Most of the species are common to the mountainous or northern and central Europe, but six cosmopolitic and three very rare ones; *Staurastrum nodosum* W. & G. S. WEST was reported from England only, *Micrasterias truncata* (CORDA) BRÉB. var. *turgida* TAYLOR was found in Newfoundland and *Micrasterias conferta* LUND was recognized as a rare northern type by many phycologists.

It is my pleasure to record here a debt of gratitude to Messrs. K. ISINO and Captain S. TAKASIMA of the Government Department of Agriculture and Forestry. Thanks are also due to Mr. S. KURAKAMI, Director of the Hokkaido Fisheries Experimental Station, and Mr. Y. SUGIURA, Director of the Imperial Fisheries Institute, for their encouragement and aid throughout this study.

The writer also wishes to express his gratitude to Prof. Dr. Y. YAMADA for his kindness extended to him in the course of this study.

# Enumeration of Desmids from the Sinsiru Island

## I. Sub-fam. SACCODERMAE

### Gen. **Spirotaenia** BRÉB. 1848.

1) **Spirotaenia condensata** BRÉB. (Pl. I, fig. 9)

YOSHI. OKADA, Desm. N. Kurile Island. 1934, p. 138, pl. 17, fig. *2*.
Length *216*$\mu$, breadth *25*$\mu$.
St. II : *2*. *not uncommon.*

### Gen. **Cylindrocystis** MENEGH. 1838.

*2*) **Cylindrocystis crassa** DE BARY. (Pl. I, figs. 15, 16)

*Cy. crassa* DE BARY. Conj. 1858, pp. 37, 74, pl. 7, figs. C 1–12; NORDST. in WITTR. & NORDST. Alg. Exsic. 1879, no. *269*; COOK. Brit. Desm. 1886, p. 46, pl. 18, fig. *2*; W. WEST, Alg. W. Ireland, 1892, p. 131; W. & G. S. WEST, Alg. N. Ireland, 1902, p. *20*; W. & G. S. WEST, Desm. Singapore, 1897, p. 156; W. & G. S. WEST, Alg. S. England, 1897, p. 478; W. & G. S. WEST, Monogr. Brit. Desm. I, 1904, p. 59, pl. 4, figs. 33–38.

*Penium crassa* WOLLE. Desm. U. S. 1884, p. 37, pl. 5, fig. 3.

Length 38-60$\mu$, breadth *22-23*$\mu$.
St. I : 4.

*Dist.*—Singapore, New Zealand, United States, England, Wales, Scotland, Ireland, France, Germany, Austria, Portugal, Sweden, Norway, Madagascar, W. Africa.
*New to Japan.*

## II. Sub-fam. PLACODERMAE

### Gen. **Penium** BRÉB. 1844.

3) **Penium didymocarpum** LUND. (Pl. I, fig. 17)

*P. didymocarpum* LUND. COOK. Brit. Desm. 1886, p. 44, pl. 17, fig. 6; W. WEST, Alg. Eng. Lake Dist. 1892, p. 721; W. G. & S. WEST, Monogr. Brit. Desm. I, 1904, p. 79, pl. 8, figs. 11–13.

Length 32 $\mu$, breadth 15 $\mu$.
St. II : *2*. *rare.*

*Dist.*—C. China, New Zealand, England, Wales, Scotland, Ireland, Austria, Sweden, Denmark, Lapland.
*New to Japan.*

4) **Penium spirospermum** Josh. (Pl. I, fig. 10)

*P. spirospermum* Josh. Notes Brit. Desm. II, 1883, p. *292*; Cook. Brit. Desm. 1886, p. 45, pl. 17, fig. 9; De Toni, Syll. Alg. 1889, p. 863; Nordst. Index Desm. 1896, p. *237*; W. & G. S. West, Monogr. Brit. Desm. I, 1904, p. 78, pl. 8, figs. 6, 7.

Length *32* $\mu$, breadth 17 $\mu$.

St. II : *2. rare.*

*Dist.*—Ceylon, England, Scotland, Ireland.

*New to Japan.*

5) **Penium spirostriolatum** Barker. (Pl. I, fig. 1)

Yoshi. Okada, *l. c.* p. 142, pl. 17, fig. 7.

Length *172* $\mu$, breadth in middle *25* $\mu$, breadth (maximum) *27* $\mu$, breadth of apices 16 $\mu$.

St. I : 3, I : 4, II : 1, II : *2*, II : 4.

## Gen. **Closterium** Nitzsch. 1817.

6) **Closterium costatum** Corda. (Pl. I, figs. 4, 5)

Yoshi. Okada, *l. c.* p. 143, pl. 18, fig. *2*.

Length 370 $\mu$, breadth 47 $\mu$.

St. I : *2*, I : 3, I : 4.

7) **Closterium Jenneri** Ralfs. (Pl. I, figs. 11, 12)

Yoshi. Okada, *l. c.* p. 146, pl. 18, fig. 9.

Length 55–80 $\mu$, breadth 8–*12* $\mu$.

St. I : 3, I : 4, II : 1, II : 3, II : 4.

8) **Closterium parvulum** Nägeli. (Pl. I, fig. 13)

Yoshi. Okada, *l. c.* p. 147, Pl. 18, fig. 7.

Length 100–*125* $\mu$, breadth 13–14 $\mu$.

St. I : 4, II : *2*, II : 4.

9) **Closterium striolatum** Ehrenb. (Pl. I, figs. *2*, 3)

Roy & Biss. Notes Jap. Desm. 1889, p. 240.

Length *289* $\mu$, brdadth *29* $\mu$.

St. II : *2*.

*Dist.*—Japan, C. China, Java, Burmah, India, Ceylon, Singapore, New Zealand, Greenland, United States, Brazil, Europe, Faeroes, Spitzbergen, Nova Zembla, E. Africa.

*New to Kuriles.*

## Gen. **Tetmemorus** RALFS. 1844.

10) **Tetmemorus granulatus** (BRÉB.) RALFS var. **attenuatus** WEST. (Pl. I, figs. 6–8)

YOSHI. OKADA, *l. c.* p. 151, pl. 20, figs. 6–7.

Length 138 $\mu$, breadth (maximum) 32 $\mu$, breadth of isthmus 28 $\mu$, breadth of apex 13 × 17 $\mu$.

St. I : 2, I : 3. *abundant.*

11) **Tetmemorus laevis** (KÜTZ.) RALFS. (Pl. I, fig. 14)

YOSHI. OKADA, *l. c.* p. 151, pl. 20, figs. 4–5.

Length 68–70 $\mu$, breadth 21–23 $\mu$, breadth of isthmus 21 $\mu$.

St. I : 2, I : 4, II : 2.

## Gen. **Euastrum** EHRENB. 1832.

12) **Euastrum aboense** ELFV. (Pl. II, figs. 7–8)

YOSHI. OKADA, *l. c.* p. 151, pl. 21, fig. 5.

Length 75 $\mu$, breadth 48–49 $\mu$, breadth of isthmus 16 $\mu$, thickness 29–32 $\mu$.

St. II : 2.

13) **Euastrum ansatum** RALFS var. **pyxidatum** DELP. (Pl. II, fig. 10)

*E. ansatum* var. *pyxidatum* DELP. Desm. subalp. 1873, p. 103, pl. 6, figs. 32–34; DE TONI, Syll. Alg. 1889, p. 1097; W. & G. S. WEST, Monogr. Brit. Desm. Vol. II, 1905, p. 29, pl. 36, figs. 14–15.

Length 78 $\mu$, breadth 41–43 $\mu$, breadth of isthmus 13 $\mu$.

St. II : 1.

*Dist.*—Poland, Italy, Scotland, Ireland.

*New to Asia.*

14) **Euastrum bidentatum** NÄGELI. (Pl. II, fig. 9)

YOSHI. OKADA, *l. c.* p. 152, Pl. 22, fig. 4.

Length 49–50 $\mu$, breadth 31 $\mu$, breadth of apex 22–25 $\mu$, breadth of isthmus 9 $\mu$.

St. I : 2, I : 4, II : 4.

15) **Euastrum binale** (TURP.) EHRENB. (Pl. II, fig. 6)

*E. binale* (TURP.) EHRENB. HASS. Brit. Freshw. Alg. 1845, p. 384, pl. 91, figs. 4, 5; RALFS, Brit. Desm. 1848, p. 90, pl. 14, figs. 8 b, e & h; DELP. Desm. subalp. 1873,

p. 107, pl. 6, figs. 9, 10; Cook. Brit. Desm. p. 75, pl. 35 figs. 6 a-d; W. West, Alg. W. Ireland, 1892, p. 139; Nordst. Index Desm. 1896, p. 59; W. & G. S. West, Alg. S. England, 1897, p. 484; W. & G. S. West, Alg. N. Ireland, 1902, p. 29; W. & G. S. West, Monogr. Brit. Desm. II, 1905, p. 51, pl. 38, figs. 28, 29.

Length 20 $\mu$, breadth 15 $\mu$, isthmus 7 $\mu$.

St. II : 2.

*Dist.*—India, Ceylon, Siam, Singapore, Australia, New Zealand, Sandwich Islands, United States, W. Indies, Brazil, Patagonia, England, Wales, Scotland, Ireland, France, Germany, Galicia & Austria, Hungary, Italy, Portugal, Norway, Sweden, Denmark, Bornholm, Finland, N. & S. Russia, Faeroes, Iceland, Nova Zembla, Spitzbergen, Greenland, Siberia, Central & E. Africa, Madagascar.

*New to Japan.*

16) **Euastrum binale** (Turp.) Ehrenb. form. **hians** West. (Pl. II, fig. 15)

*E. binale* form. *hians* West. Alg. W. Ireland, 1892, p. 140, pl. 20, fig. 14; W. & G. S. West, Monogr. Brit. Desm. II, 1905, p. 53, pl. 38, fig. 33.

Length 13 $\mu$, breadth 12 $\mu$, breadth of isthmus 6 $\mu$.

St. II : 2.

*Dist.*—Ceylon, England, Ireland, Germany.

*New to Japan.*

17) **Euastrum crassum** (Bréb.) Kütz. (Pl. II, figs. 13, 14)

Yoshi. okada, *l. c.* p. 153, pl. 22, figs. 1, 2.

Length 162 $\mu$, breadth 85 $\mu$, breadth of isthmus 28 $\mu$, breadth of apex 48–50 $\mu$; thickness 63–65 $\mu$.

St. II : 2.

18) **Euastrum Didelta** (Turp.) Ralfs. (Pl. II, fig. 16)

Yoshi. Okada, *l. c.* p. 153, pl. 22, fig. 3.

Length 119 $\mu$, breadth 68–69 $\mu$, breadte of isthmus 22 $\mu$.

St. I : 2, I : 3, I : 4, II : 4.

19) **Euastrum obesum** Josh. (Pl. III, fig. 3)

Yoshi. Okada, *l. c.* p. 155, pl. 23, fig. 1.

Length 66 $\mu$, breadth 47 $\mu$, breadth of apex 18 $\mu$, breadth of isthmus 13 $\mu$.

St. I : 3, I : 4, II : 2, II : 3, II : 4.

20) **Euastrum oblongum** (Grev.) Ralfs. (Pl. II, figs. 1–3)

Yoshi. Okada, *l. c.* p. 155, pl. 21, fig. 7.

Length 131 $\mu$, breadth 70–72 $\mu$, breadth of isthmus 25 $\mu$, breadth of polar lobe 38–40 $\mu$.

St. I : 4, II : 1, II : 2, II : 3, II : 4. *abundant.*

21) **Euastrum pinnatum** RALFS. (Pl. II, figs. 11–12)

YOHSI. OKADA, *l. c.* p. 156, pl. 24, fig. 5.

Length 58 $\mu$ (semicell), breadth 63 $\mu$, baeadth of isthmus 20 $\mu$, breadth of polar lobe 33 $\mu$, thickness 28 $\mu$.

St. II : 1, II : 2.

22) **Euastrum sublobatum** BRÉB. (Pl. II, figs. 4, 5)

YOSHI. OKADA, *l. c.* p. 157, pl. 23, fig. 7.

Length 26 $\mu$, breadth 21 $\mu$, breadth of apex 16–17.5 $\mu$, breadth of isthmus 7 $\mu$.

St. I : 2, II : 2, II : 4.

23) **Euastrum verrucosum** EHRENB. var. **alatum** WOLLE. (Pl. II, fig. 17)

*E. verrucosum* var. *alatum* WOLLE. Desm. U. S. 1884, p. 101, pl. 26, fig. 4; DE TONI, Syll. Alg. 1889, p. 1067; W. WEST, Alg. W. Ireland, 1892, p. 136; W. & G. S. WEST, Monogr. Brit. Desm. II, 1905, p. 67, pl. 40, fig. 6.

Length 80 $\mu$, breadth 72–75 $\mu$, breadth of isthmus 20 $\mu$, breadth of polar lobe 32–37 $\mu$, thickness 45 $\mu$.

St. I : 4, II : 1, II : 4.

*Dist.*—United States, Scotland, Ireland, Galicia in Austria, Sweden, N. Russia.

*New to Asia.*

## Gen. **Micrasterias** AG. 1827.

24) **Micrasterias conferta** LUND. (Pl. IV, fig. 5)

*M. conferta* LUND. Desm. Suec. 1871, p. 14, pl. 1, fig. 5; WOLLE, Desm. U. S. 1884, p. 114, pl. 53, figs. 12, 13; COOKE, Brit. Desm. 1886, p. 59, pl. 28, fig. 2; DE TONI, Syll. Alg. 1889, p. 1128; W. & G. S. WEST, Scott. Freshw. Plankton, 1, 1903, p. 525; W. & G. S. WEST, Monogr. Brit. Desm. II, 1905 p. 88, pl. 43, figs. 4–8; TAYLOR, Freshw. Alg. Newfoundland. II, 1935, p. 212, pl. 46, fig. 3.

Length 108 $\mu$, breadth 105–103 $\mu$, breadth of isthmus 22 $\mu$, breadth of polar lobe 38–40 $\mu$.

St. II : 2. *very rare.*

*Dist.*—United States, Scotland, France, Norway, Sweden, Finland, Greenland.

*New to Asia.*

The present species is essentially of a northern type and it is one of the rarest Desmids in Japan.

25) **Micrasterias denticulata** Bréb. var. **angulosa** (Hantzsch) W. & G. S. West. (Pl. III, figs. 1–2)

Yoshi. Okada, *l. c.* p. 159, pl. 21, fig. 1.

Length 218 $\mu$, breadth 187 $\mu$, breadth of isthmus 31 $\mu$, breadth of polar lobe (maximum) 60 $\mu$.

St. II : 2.

26) **Micrasterias papillifera** Bréb. (Pl. III, fig. 4; Pl. IV, figs. 11, 12)

Yoshi. Okada, *l. c.* p. 160, pl. 25, figs. 1, 2.

Length 128 $\mu$, breadth 112–116 $\mu$, breadth of isthmus 18–20 $\mu$, breadth of polar lobe 42 $\mu$.

St. I : 3, II : 2.

27) **Micrasterias Thomasiana** Arch. (Pl. IV, fig. 1)

Yoshi. Okada, *l. c.* p. 162, pl. 22, figs. 5, 6.

Length 198 $\mu$, breadth 177 $\mu$, breadth of isthmus 30 $\mu$, breadth of polar lobe 47–51 $\mu$.

St. II : 2.

28) **Micrasterias truncata** (Corda) Bréb. var. **turgida** Taylor. (Pl. III, figs. 5–7)

*M. truncata* var. *turgida* Taylor. Freshw. Alg. Newfoundland. II, 1935, p. 216, pl. 46, figs. 6–8, pl. 47, figs. 2–3.

*M. truncata* (Corda) Bréb. Yoshi. Okada *l. c.* p. 162, pl. 24, figs. 1, 2.

Length 95 $\mu$, breadth 82–85 $\mu$, breadth of isthmus 20 $\mu$, thickness 42–48 $\mu$.

St. I : 3, II : 2.

*Dist.*—Northern Kuliles, Newfoundland.

*New to Asia.*

## Gen. **Cosmarium** Corda. 1834.

29) **Cosmarium caelatum** Ralfs var. **spectabile** (De Not.) Nordst. (Pl. V, fig. 7)

Yoshi. Okada, *l. c.* p. 164, pl. 27, fig. 13.

Length 61 $\mu$, breadth 47 $\mu$, breadth of isthmus 13 $\mu$.

St. I : 2, I : 3, I : 4, II : 4.

30) **Cosmarium contractum** KIRCHN. var. **ellipsoideum** (ELFV.) W. & G. S. WEST. (Pl. IV, figs. 9, 10).

YOSHI. OKADA, *l. c.* p. 164, pl. 27, fig. 8.

Length 26–27 $\mu$, breadth 23–24 $\mu$, breadth of isthmus 9–11 $\mu$.

St. II : 2.

31) **Cosmarium dentiferum** CORDA. (Pl. V, figs. 1–2)

*C. dentiferum* CORDA. NORDST. Index Deam. 1896, p. 101; W. & G. S. WEST, Monogr. Brit. Desm. III, 1908, p. 156, pl. 78, figs. 17, 18.

Length 62 $\mu$, breadth 51–55 $\mu$, breadth of isthmus 20 $\mu$.

St. I : 4, II : 4.

*Dist.*—England, Scotland, Germany, Bornholm, Iceland.

*New to Asia.*

32) **Cosmarium impressulum** ELFV. (Pl. V, fig. 9)

YOSHI. OKADA, *l. c.* p. 167, pl. 26, fig. 6.

Length 28 $\mu$, breadth 22 $\mu$, breadth of isthmus 7 $\mu$.

St. II : 2.

33) **Cosmarium nasutum** NORDST. form. **granulata** NORDST. (Pl. IV, figs. 6, 7)

YOSHI. OKADA, *l. c.* p. 168, pl. 27, fig. 4.

Length 63 $\mu$, breadth 48–50 $\mu$, breadth of isthmus 21 $\mu$, thickness 22 $\mu$.

St. I : 2, I : 3, I : 4, II : 4.

34) **Cosmarium ornatum** RALFS. (Pl. V, figs. 3, 4)

*C. ornatum* RALFS. HASS. Brit. Freshw. Alg. 1845, p. 364, pl. 86, fig. 3; RALFS, Brit. Desm. 1848, p. 104, pl. 16, fig. 7; WOLLE, Desm. U. S. 1884, p. 82, pl. 18, figs. 39–42, pl. 49, figs. 23, 24; COOKE, Brit. Deam. 1887, p. 112, pl. 40, fig. 4; DE TONI, Syll. Alg. 1889, p. 1025; W. WEST, Alg. W. Ireland, 1892, p. 158; W. WEST, Alg. Eng. Lake Dist. 1892, p. 728; NORDST. Index Desm. 1896, p. 190; W. & G. S. WEST, Alg. S. England, 1902, p. 40; W. & G. S. WEST, Scott. Freshw. Plankt. I, 1903, p. 527; BORGE, Alg. Erst. Regnell. Exped. II, Desm. 1903, p. 100; W. & G. S. WEST, Comp. Study Plankt. Irish Lakes, 1906, p. 85; W. & G. S. WEST, Monogr. Brit. Desm. III, 1908, p. 151, pl. 78, figs. 1–10.

Length 40 $\mu$, breadth 38 $\mu$, breadth of isthmus 15 $\mu$, thickness 26 $\mu$.

St. II : 2.

*Dist.*—United States, Newfoundland, Brazil, Paraguay, England, Wales, Scotland, Ireland, France, Germany, Austria, Poland, Italy, Portugal, Norway, Sweden, Bornholm, Denmark, Finland, N. & S. Russia, Iceland.

*New to Asia.*

35) **Cosmarium praemorsum** Bréb. (Pl. IV, figs. 2–4)

*C. praemorsum* Bréb. Cooke, Brit. Desm. 1887. p. 107, pl. 42, fig. 2; De Toni, Syll. Alg. 1889, p. 1000; W. West, Alg. W. Ireland, 1892, p. 155; W. West, Alg. Eng. Lake Dist. 1892, p. 728; Nordst. Index Desm. 1896, p. 205; W. & G. S. West, Alg. S. England, 1897, p. 490; Börg. Freshw. Alg. Faeroés, 1901, p. 225; W. & G. S. West, Alg. N. Ireland, 1902, p. 39; Borge, Beiträge Alg. Schweden, 1906, p. 31; W. & G. S. West, Monogr. Brit. Desm. III, 1908, p. 196, pl. 84, figs. 1–5.

Length 55 $\mu$, breadth 43–47 $\mu$, breadth of isthmus 18 $\mu$, thickness, with granules, 36 $\mu$.

St. II : 2.

*Dist.*—England, Wales, Scotland, Ireland, France, Germany, Switzerland, Austria, Norway, Sweden, Faeroes, Finland, Russia, E. Africa.

*New to Asia.*

36) **Cosmarium quadrifarium** Lund. form. **hexasticha** (Lund.) Nordst. (Pl. IV, fig. 8)

Yoshi. Okada, *l. c.* p. 171, pl. 27, figs. 6–6′.

Length 47 $\mu$, breadth 37 $\mu$, breadth of isthmus 20 $\mu$.

St. I : 4, II : 2, II : 4.

## Gen. **Xanthidium** Ehrenb. 1837.

37) **Xanthidium antilopaeum** (Bréb.) Kütz. (Pl. V, figs. 5, 6)

Yoshi. Okada, *l. c.* p. 174, pl. 28, fig. 1.

Length, without spines, 51 $\mu$, with spines 73 $\mu$; breadth, without spines, 52–55 $\mu$, with spines 80–85 $\mu$; breadth of isthmus 17 $\mu$; thickness 31 $\mu$.

St. II : 2.

38) **Xanthidium armatum** (Bréb.) Rabenh. (Pl. V, fig. 10)

Yoshi. Okada, *l. c.* p. 175, pl. 28, figs. 12–12″.

Length, without spines, 125 $\mu$, with spines 132 $\mu$; breadth, without spines, 85 $\mu$, with spines 103–105 $\mu$; breadth of isthmus 38 $\mu$.

St. II : 2.

## Gen. **Arthrodesmus** EHRENB. 1838.

39) **Arthrodesmus convergens** EHRENB. (Pl. V, figs. 13–14)

YOSHI. OKADA, *l. c.* p. 177, pl. 28, figs. 2, 3.

Length 45 $\mu$; breadth, without spines, 49–55 $\mu$, with spines 66–71 $\mu$; breadth of isthmus 15 $\mu$; length of spines 7–10 $\mu$; thickness 28 $\mu$.

St. II : 2.

## Gen. **Staurastrum** MEYEN. 1829; em. RALFS.

40) **Staurastrum nodosum** W. & G. S. WEST. (Pl. V, fig. 8)

*St. nodosum* W. & G. S. WEST, Alg. S. England, 1897, p. 495, pl. 6, fig. 23; W. & G. S. WEST & CARTER, Monogr. Brit. Deam. V, 1923, p. 88, pl. 141, fig. 16.

Length, without processes, 15 $\mu$, with processes, 20 $\mu$; breadth, with processes, 20 $\mu$; breadth of isthmus 7 $\mu$.

St. II : 2.

*Dist.*—England.

*New to Asia.*

41) **Staurastrum orbiculare** RALFS. (Pl. V, figs. 15–16)

*St. orbiculare* RALFS. Brit. Desm, 1848, p. 125, pl. 21, fig. 5h; DELP. Desm. subalp. 1877, p. 37, pl. 10, fig. 11; W. & G. S. WEST, Monogr. Brit. Desm. IV, 1912, p. 155, pl. 124, figs. 10, 11.

*St. hibernicum* WEST var. *Farquharsonii* (ROY & BISS.) W. & G. S. WEST, Notes Alg. II, 1900, p. 296, pl. 412, fig. 21.

Length 45 $\mu$, breadth 32–35 $\mu$, breadth of isthmus 11 $\mu$.

St. II : 2.

*Dist.*—England, Scotland, Germany, Sweden, Finland, Italy.

*New to Asia.*

42) **Staurastrum teliferum** RALFS. (Pl. V, figs. 11, 12)

YOSHI. OKADA, *l. c.* p. 190, pl. 30, fig. 3.

Length, without spines, 45 $\mu$, with spines, 52 $\mu$; breadth, without spines, 38 $\mu$, with spines, 47 $\mu$; breadth of isthmus 12 $\mu$.

St. II : 4.

## Gen. **Hyalotheca** Ehrenb. 1840.

43) **Hyalotheca dissiliens** (Sm.) Bréb. (Pl. V, figs. 17~20)
Yoshi. Okada, *l. c.* p. 192, pl. 31, fig. 2.
Length 14–20 $\mu$, breadrh 20–30 $\mu$.

form. **minor** Delp. (fig. 17)

form. **tridentula** Nordst. (fig. 18)

form. **bidentula** Nordst. (fig. 19)
St. I : 4, II : 2.

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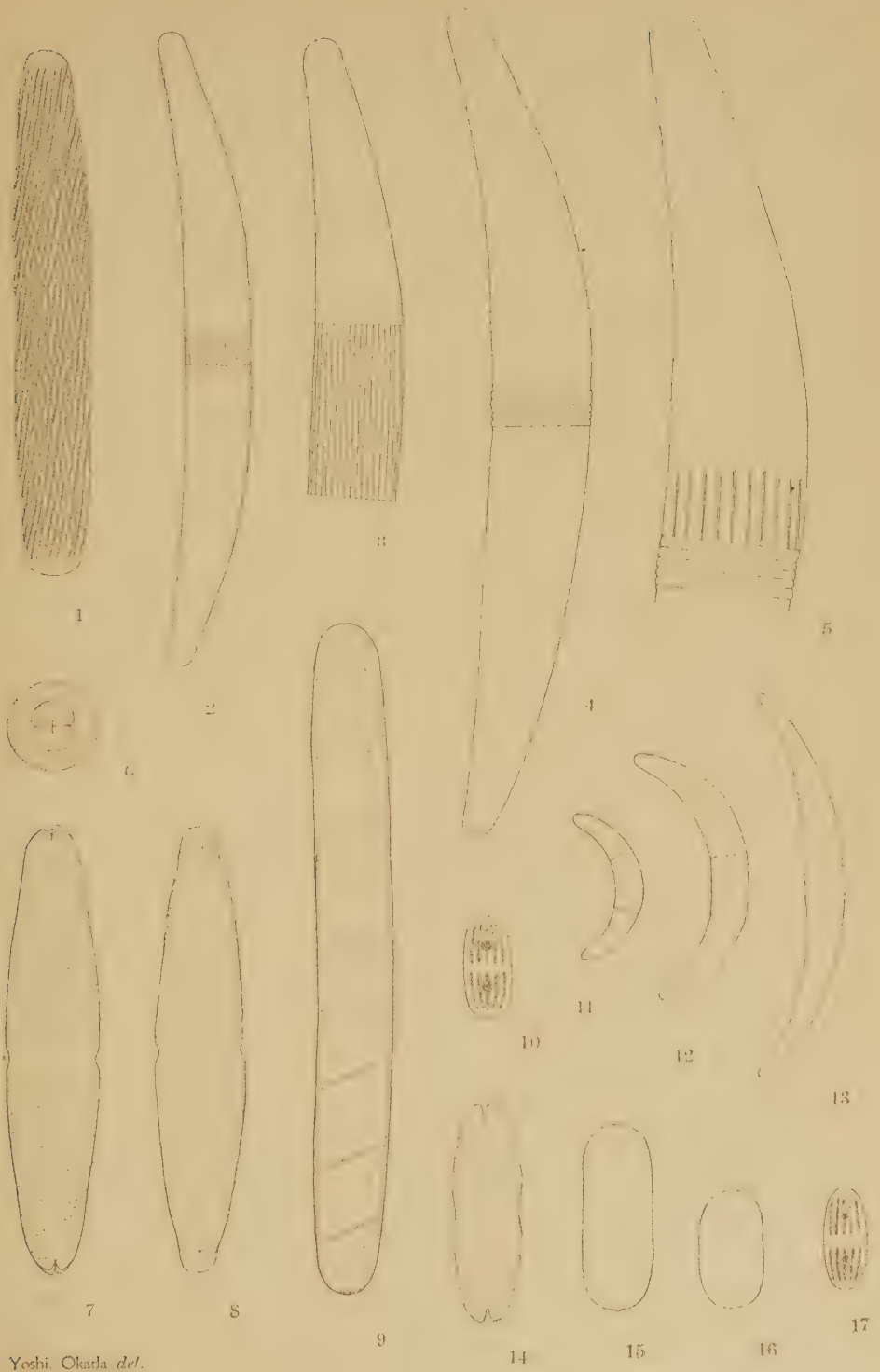
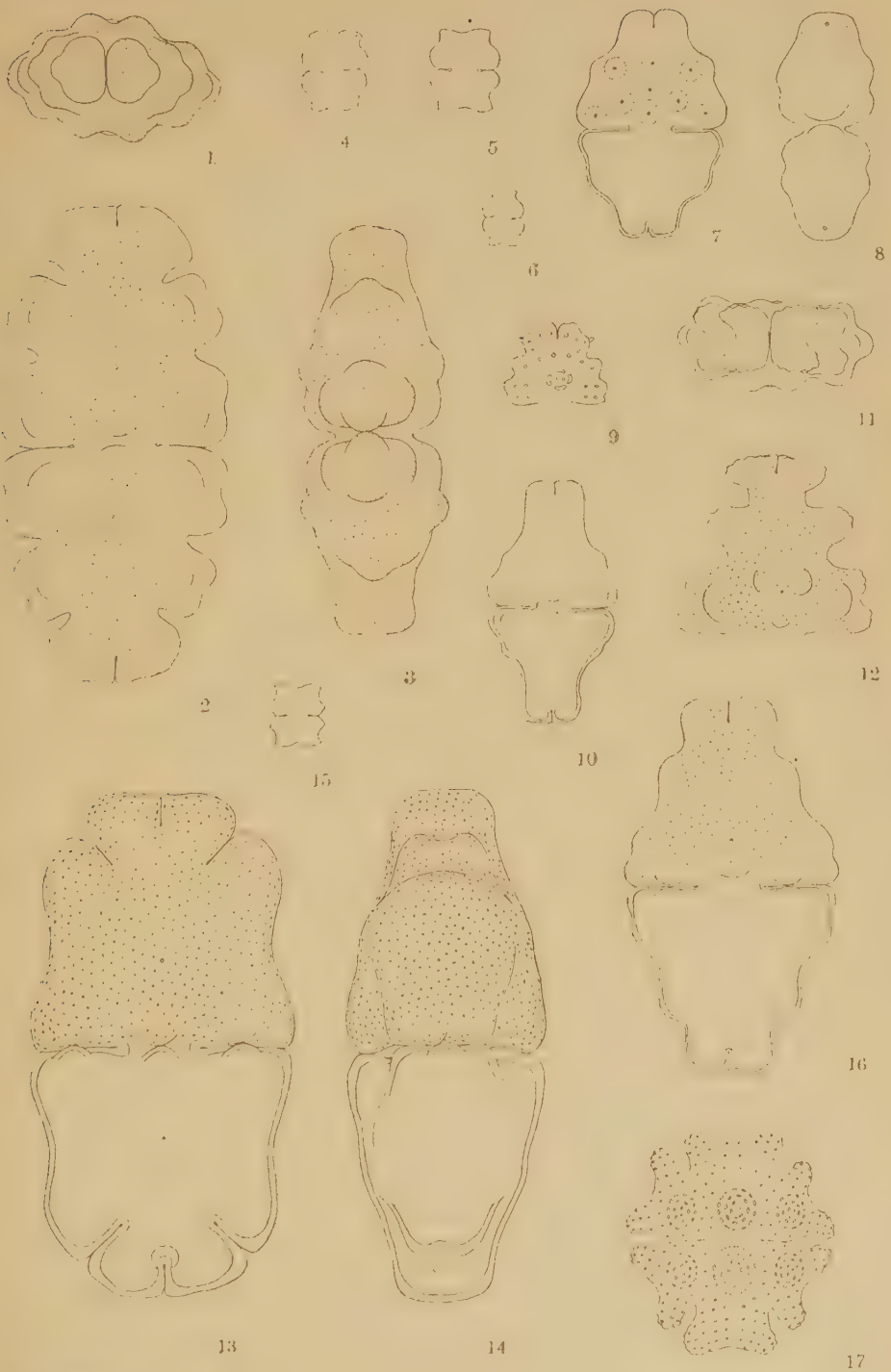



Yoshi. Okada *del.*

Desmids from Sinsiru Island

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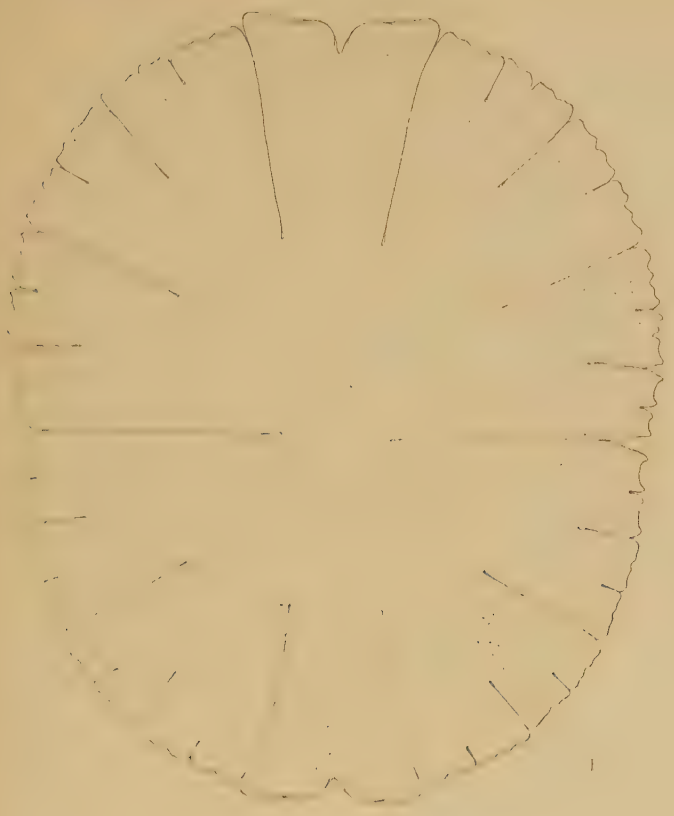

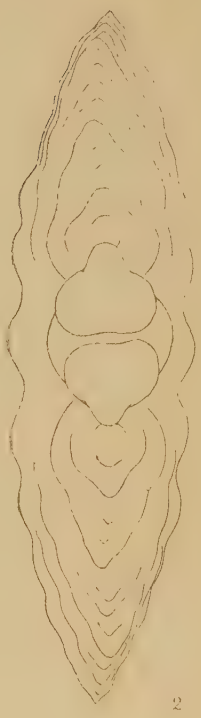

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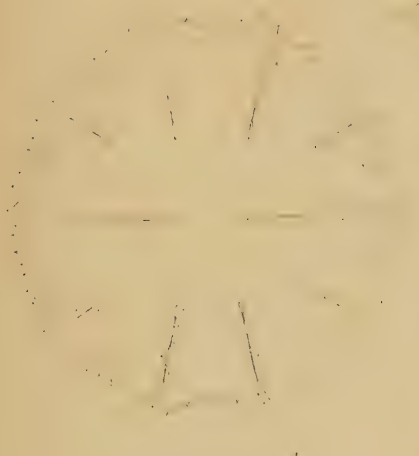

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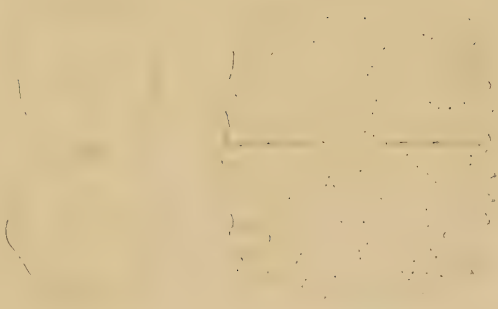

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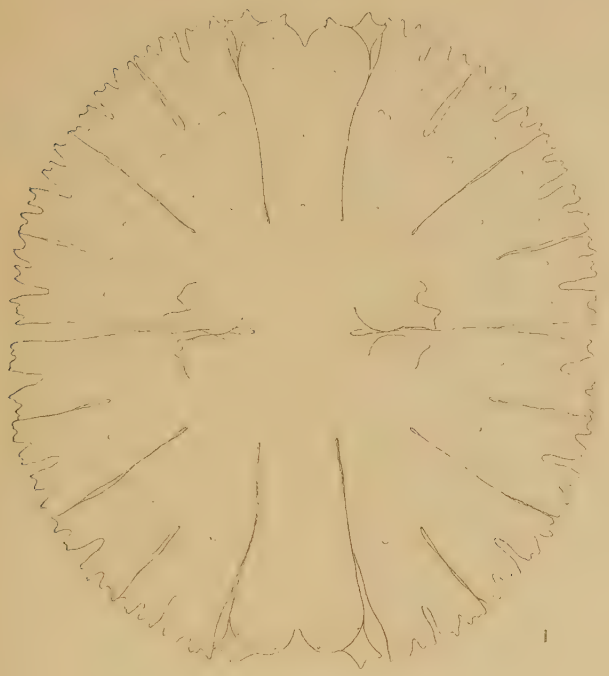
Desmids from Sinsiru Island



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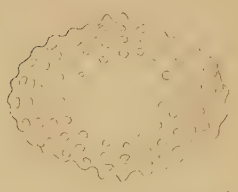

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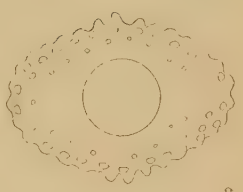

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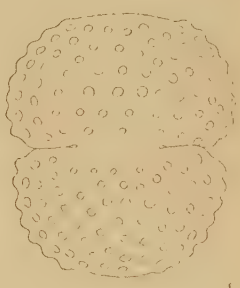

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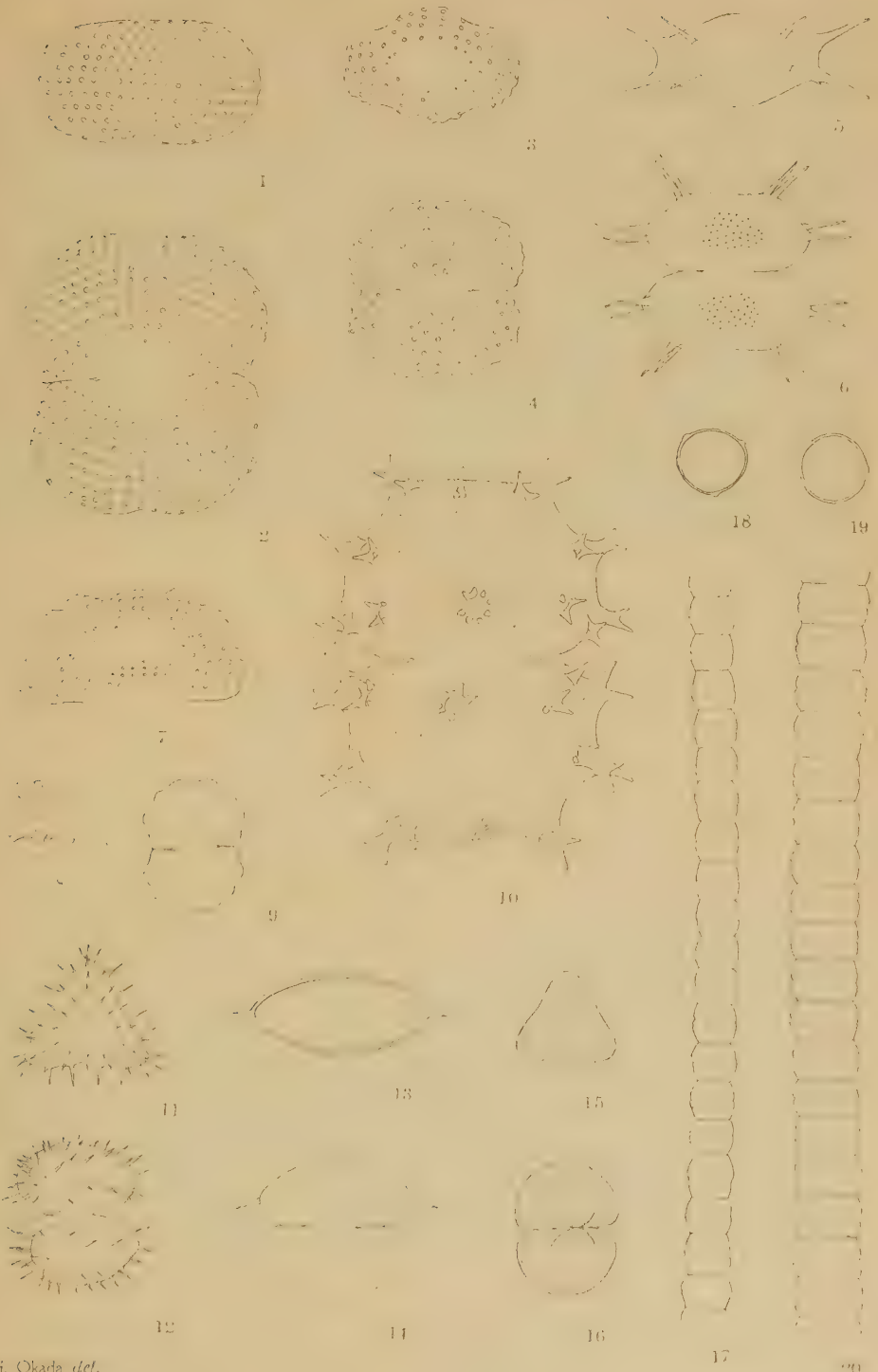
Desmids from Sinsiru Island

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Desmids from Sinsiru Island

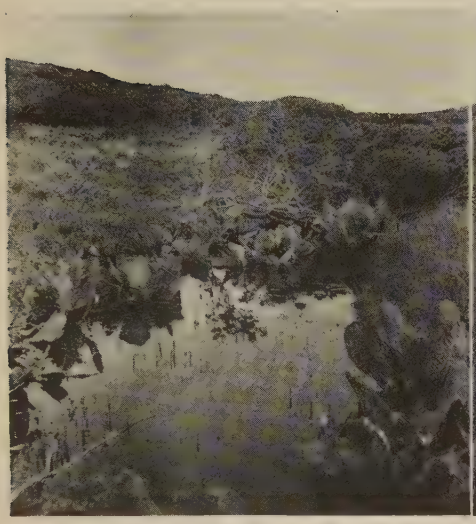

Fig. 1

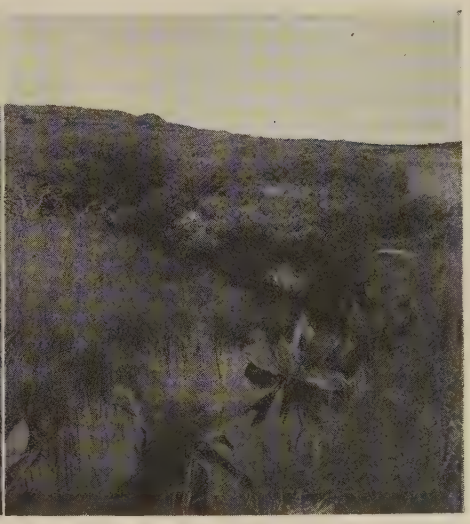

Fig. 2

**Fig. 1.** St. II. One of the pools in the boglet at the western foot of Mt. Mikazuki. Dwarf alpine pine, *Pimus pumila* Regel, and *Lysichiton camtschatense* Schott are seen on the border.

**Fig. 2.** St. II. Another pool at the western foot of Mt. Mikazuki. *Pinus pumila* Regel and *Betula Ermanii* Cham. var. *communis* Koidz. which are usually found in the alpine region of Japan Proper, are seen growing among the swampy grass, *Lysichiton camtschatense* Schott.

Fig. 3

Fig. 4

**Fig. 3.** St. I. One of the pools in the eastern part of Mt. Mikazuki. *Pinus pumila* Regel is seen on the border.

**Fig. 4.** St. II. One of the pools in the western part of Mt. Mikazuki. Shrubs on the border are *Pinus pumila* Regel and *Sorbus sambucifolia* M. Roem.

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January, 1939

# STUDIES ON SOME PHYSICO-CHEMICAL PROPERTIES AND THE METHODS FOR ISOLATION AND DETERMINATION OF CERTAIN AMINO ACIDS

Tetuo TOMIYAMA

(Received for publication, May 18, 1938)

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# INTRODUCTION

Since the amino acids were discovered as constituents of proteins, chemical studies of them have been made to such an extent that even an historical sketch would require a monograph of large volume. It should be noted that EMIL FISCHER and his pupils, amongst others, have produced numerous excellent works on the method of isolation as well as on the structure of amino acids.

The physico-chemical properties of amino acids, however, had been left untouched until recent time, when they were attacked by several American investigators, such as CARL L. A. SCHMIDT, E. J. COHN, *et al.* Many interesting facts have been brought out from these recent works, while there are many physico-chemical constants which are still left to be found. Moreover, though many studies have been made on the method of isolation and determination of amino acids, there is almost none which is considered perfect.

The present dissertation deals, in the first part, with the studies of some physico-chemical properties, and in the second part, with the examination of the method of isolation and determination of several amino acids. In the first part, some newly obtained physico-chemical constants are reported, and their theoretical significances are discussed. In the second part, descriptions are made of some improvements of several methods, *e. g.*, the VAN SLYKE analysis, the method for determining tryptophane, etc.

The studies have been carried out under the direction of PROF. Y. OKUDA and DR. M. KITAGAWA of the Kyushu Imperial University, to whom the author is indebted for helpful advice. The author wishes to express his thanks to PROF. CARL L. A. SCHMIDT of the University of California for his suggestion kindly given on the determination of solubility and apparent dissociation constant. Thanks are also due to DR. R. BRDIČKA of the Charles University, Prag, Czechoslovakia, for his helpful criticisms in regard to the problems discussed in Chapter II. The author is also greatly indebted to MR. S. SIGEMATU, MR. K. KASIWADA, MR. T. HARADA, MR. K. KATAI, and MR. M. HANADA for their assistance in carrying out some of the studies.

# PART I

# STUDIES ON SOME PHYSICO-CHEMICAL PROPERTIES OF CERTAIN AMINO ACIDS

## CHAPTER I

## The Apparent Dissocation Constants of Canavanine and Canaline

KITAGAWA and TOMIYAMA[1] found a new basic compound, canavanine, in jack bean meal and showed that, in certain respects, it possesses a behavior somewhat similar to that of arginine. However, when canavanine is split by liver ferment, it does not give rise to urea and ornithine, but to urea and canaline. From its chemical behavior the latter compound is believed to be $\alpha$-amino-$\gamma$-hydroxyamino butyric acid.[2] KITAGAWA and YAMADA[3] reported experimental evidence to the effect that canavanine is not a ureido compound, but may be a guanidine compound. The tentative formulæ of canavanine and canaline are as follows:

Canavanine

Canaline

In order to throw light on the structure of canavanine and its decomposition product, the apparent dissociation constants of the two compounds have been determined, since the value of the dissociation constant corresponding to any particular group is considered to be a function both of the position of that group and the influence of adjoining groups in the molecule.

### Experimental

(1) *The preparation of canavanine.*

Canavanine was isolated from Arlco jack been meal by KITAGAWA and YAMADA's method.[3] The meal was extracted with 50 per cent alcohol three times as much as the weight of the material. To the extract, after being evaporated under reduced pressure, was added 95 per cent alcohol, thus precipitating most of canavanine. The dissolution of the precipitate in water and the precipitation of

crude canavanine by adding 95 per cent alcohol were repeated several times, and to the aqueous solution was added an excess of flavianic acid. The canavanine flavianate thus obtained was further converted into dipicrate by removing flavianic acid with butyl alcohol and adding a small excess of picric acid enough to make canavanine dipicrate. The dipicrate was recrystallized from water several times. The nitrogen content of the canavanine picrate was determined as 21.87 per cent (calculated for $C_5H_{12}O_3N_4 \cdot 2\,C_6H_3N_3O_7$, N = 22.08 %). For the determination of dissociation constant was used canavanine sulfate solution which was prepared from the dipicrate by adding an excess of sulfuric acid, extracting the picric acid with ether and further removing the excess of sulfuric acid with baryta.

(2) *The preparation of canaline.*

The canavanine sulfate solution which had been prepared by the above method, was hydrolyzed for three days at pH 7.5 by a well-washed suspension of pig liver obtained by KITAGAWA and YAMADA'S method.[3] From the hydrolyzate, canaline was isolated as dipicrate and purified several times by recrystallization. The nitrogen content of the canaline picrate was 18.90 per cent (calculated for $C_4H_{10}O_3N_2 \cdot 2\,C_6H_3N_3O_7$, N = 18.92 %). The canaline sulfate solution, prepared just as described in the paragraph (1), was used for the determination of the dissociation constant.

(3) *The method for the determination of dissociation constant.*

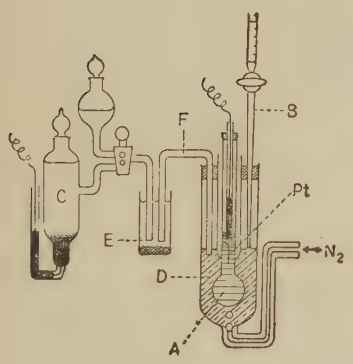
The dissociation constant was calculated on the basis of the amount of caustic soda added as well as the resulting pH of the solution. A difficulty arose in this titration procedure since it was impossible to use either the hydrogen or the quinhydrone electrode. Canaline is easily decomposed by hydrogen in the presence of platinum black and in the case of canavanine it was found that both the hydrogen and quinhydrone electrode showed unsteady potentials soon after starting to neutralize the $\alpha$-amino group, i.e., the pH region above pH 4. Hence the glass electrode shown in Fig. 1 was used. The glass electrode was made of Corning glass 015. The inner part of the glass bulb was filled with acetate buffer which was saturated with quinhydrone and in which was dipped a platinum electrode. The glass electrode was dipped into a sample solution which was electrically connected with a saturated calomel cell. The potential difference between the platinum electrode and the calomel electrode was measured by a vacuum tube potentiometer of DE EDS type[4] using a galvanometer, sensitivity of which was $10^{-8}$ amp/mm.

Adopting pH of the acetate buffer 4.62 as a standard, pH of the amino acid solution was calculated by the following equation:

$$\mathrm{pH} = \frac{E_s \sim E_a}{0.0581 + 0.0002\,(t - 20)} + 4.62$$

where, $E_s$ = the potential difference measured when the glass electrode is dipped in a sample solution,

$E_a$ = the potential difference measured when the glass electrode is dipped in the acetate buffer.




A Acetate buffer saturated with quinhydrone.
B Micro burette.
C 1/10 *N* Calomel electrode.
D Sample solution.
E Saturated solution of potassium chloride.
F Agar bridge.
Pt Blank platinum electrode.

Fig. 1. The apparatus for the titration experiment

The glass electrode was calibrated with certain buffers having known pH, and was found to behave between pH 2 and pH 10 exactly as the hydrogen electrode. The accuracy of the measurement was ± 0.01 pH in the pH region where the glass electrode functioned normally. Corrections were made above pH 10. The titration experiments were carried out as follows: 10 cc of about 0.02 *M* solution of the amino acid sulfate were taken in a titration vessel (Fig. 1) and titrated with 1.00 *M* sodium hydroxide using a micro burette, the solution being stirred by bubbling nitrogen gas through it. The maximum change of volume during the titration was about 6 per cent. Such a small extent of dilution may be assumed to be no effect on the dissociation constant. All the measurements were carried out at 25°.

## Experimental Results and Discussions

By using the Henderson-Hasselbalch equations (1) and (2), the negative loga-

rithmic value for the apparent dissociation constant pK′ was obtained, averaging the values which were calculated from pH value and number of mols of caustic soda added at each titration point.[5]

$$pK'_a = pH + \log \frac{C_a - C_{B^+} + C_{OH^-}}{C_{B^+} - C_{OH^-}} \quad (1)$$

$$pK'_b = pK_w - pH + \log \frac{C_a - C_{A^-} + C_{H^+}}{C_{A^-} - C_{H^+}} \quad (2)$$

where, $C_a$ = the molar concentration of amino acid.

$C_{B^+}$ = mols of caustic soda added.

$C_{A^-}$ = mols of acid added.

$C_{OH^-}$ = the concentration of hydroxyl ion in the solution which is equivalent to the amount of hydrolyzed part of sodium salt of amino acid.

$C_{H^+}$ = the concentration of hydrogen ion which is equivalent to the amount of hydrolyzed part of amino acid sulfate.

$pK_w$ = the dissociation constant of water, i.e., 14.00 at 25°.

Different values for the apparent acid and base dissociation constant have been ascribed to the acidic and basic groups of diamino acids, on the assumption that whether amino acids exist in zwitter ion or unionized form. It has been remained unknown that which basic group of diamino acid forms zwitter ion. However, the dissociation constant for zwitter ion can be readily calculated from the classical dissociation constant, hence the latter form has been used throughout the present paper. According to the classical view of the dissociation, ionization of amino acid by the addition of acid or base takes place as shown in equations (3) and (4).

$$H_2N{-}R{-}COOH + HA \rightleftarrows H_3N^+{-}R{-}COOH + A^- \quad (3)$$

$$H_2N{-}R{-}COOH + BOH \rightleftarrows H_2N{-}R{-}COO^- + B^+ + H_2O \quad (4)$$

Experimental results are shown in Tables I and II, and the titration curves of canavanine and canaline are shown in Figs. 2 and 3. The average values for these dissociation constants are shown in Table III. From these constants, the apparent free energy change $\Delta F'_{298}$ due to the ionization[6] was calculated by equation (5). The isoelectric point of canavanine and canaline was also calculated from values for $pK'_a$, $pK'_{b_1}$, and $pK'_{b_2}$, by equation (6).

$$\Delta F'_{298} = -RT' \ln K' \quad (5)$$

$$pI = \frac{1}{2}\left\{pK'_a + pK_w + \log (K'_{b_1} + K'_{b_2})\right\} \quad (6)$$

TABLE I

*Calculation of pK′ from the Titration Values of 0.022 M Canavanine.*

| Amount of 1.02 *M* NaOH added | Mols of acid or base combined | Corrected pH | $pK_w - pH$ | $C_{H^+}$ or $C_{OH^-}$ | † *A* or †† *B* | pK′ | |
|---|---|---|---|---|---|---|---|
| 0.040 | 0.417 (0.180)* | 1.78 | 12.22 | $C_{H^+}$ $1.66\times10^{-2}$ | *A* | | |
| 0.102 | 0.383 (0.150)* | 1.87 | 12.13 | 1.35 〃 | −0.11 | | |
| 0.152 | 0.382 (0.100)* | 2.00 | 12.00 | 1.00 〃 | −0.25 | 11.75 | |
| 0.202 | 0.356 (0.075)* | 2.17 | 11.83 | $6.76\times10^{-3}$ 〃 | −0.33 | 11.50 | $pK'_{b_2}$ |
| 0.251 | 0.328 (0.055)* | 2.30 | 11.70 | 5.01 〃 | −0.23 | 11.47 | |
| 0.301 | 0.287 (0.044)* | 2.50 | 11.50 | 3.16 〃 | −0.14 | 11.34 | |
| 0.351 | 0.263 (0.018)* | 2.81 | 11.19 | 1.55 〃 | −0.11 | 11.09 | |
| 0.381 | 0.244 (0.006)* | 3.17 | 10.83 | $6.76\times10^{-4}$ 〃 | | | |
| 0.401 | 0.230 | 3.86 | 10.14 | 1.33 〃 | | | |
| 0.421 | 0.209 | 5.85 | 8.15 | $1.41\times10^{-6}$ 〃 | | | |
| 0.441 | 0.189 | 6.33 | 7.67 | $4.68\times10^{-7}$ 〃 | *A* +0.12 | 7.79 | |
| 0.481 | 0.148 | 6.78 | 7.22 | 1.66 〃 | +0.29 | 7.51 | $pK'_{b_1}$ |
| 0.552 | 0.074 | 7.32 | 6.68 | $4.79\times10^{-8}$ 〃 | +0.69 | 7.37 | |
| 0.584 | 0.042 | 7.61 | 6.39 | 2.45 〃 | +0.98 | 7.37 | |
| 0.604 | 0.021 | 7.84 | 6.16 | 1.45 〃 | +1.30 | 7.46 | |
| 0.625 | 0.000 | 8.10 | | | | | |
| 0.646 | 0.021 | 8.40 | 5.60 | $C_{OH^-}$ $2.51\times10^{-6}$ | *B* +0.97 | 9.37 | |
| 0.687 | 0.063 | 8.88 | 5.12 | 7.59 〃 | +0.40 | 9.28 | $pK'_a$ |
| 0.728 | 0.105 | 9.24 | 4.76 | $1.74\times10^{-5}$ 〃 | +0.04 | 9.28 | |
| 0.768 | 0.146 | 9.57 | 4.43 | 3.72 〃 | −0.29 | 9.28 | |
| 0.808 | 0.187 | 10.00 | 4.00 | $1.00\times10^{-4}$ 〃 | −0.74 | 9.26 | |
| 0.828 | 0.206 | 10.26 | 3.74 | 1.82 〃 | −1.06 | 9.20 | |

* Blank for distilled water.

† $A = \log \dfrac{C_a - C_{A^-} + C_{H^+}}{C_{A^-} - C_{H^+}}$ †† $B = \log \dfrac{C_a - C_{B^+} + C_{OH^-}}{C_{B^+} - C_{OH^-}}$

TABLE II

*Calculation of pK′ from the Titration Values of 0.024 M Canaline.*

| Amount of 1.02 *M* NaOH added | Mols of acid or base combined | Corrected pH | $pK_w$−pH | $C_{H^+}$ or $C_{OH^-}$ | † *A* or †† *B* | pK′ |
|---|---|---|---|---|---|---|
| | | | | $C_{H^+}$ | *A* | |
| 0.050 | 0.313 (0.075)* | 2.16 | 11.84 | $6.92\times10^{-3}$ | −0.04 | 11.80 |
| 0.081 | 0.300 (0.056)* | 2 23 | 11.71 | 5 13 〃 | −0.03 | 11 68 |
| 0.102 | 0.285 (0.050)* | 2.36 | 11.64 | 4.36 〃 | +0.00 | 11.64 |
| 0.122 | 0.274 (0.040)* | 2.46 | 11.54 | 3.47 〃 | +0.00 | 11.54 $pK'_{b_2}$ |
| 0.142 | 0.264 (0.030)* | 2.59 | 11.41 | 2.57 〃 | +0.00 | 11.41 |
| 0.162 | 0.253 (0.020)* | 2.73 | 11 27 | 1.86 〃 | +0.02 | 11.29 |
| 0.182 | 0.239 (0.014)* | 2.90 | 11.10 | 1.26 〃 | | |
| 0.202 | 0.225 (0.008)* | 3.08 | 10.92 | $8.32\times10^{-4}$ | | |
| 0.222 | 0.208 (0.004)* | 3.28 | 10.72 | 5.25 〃 | | |
| 0.241 | 0.191 (0.002)* | 3.46 | 10.54 | 3.47 〃 | | |
| 0.261 | 0.172 | 3.65 | 10.35 | 2.24 〃 | *A* | |
| 0.281 | 0.153 | 3.79 | 10.21 | 1.62 〃 | +0.34 | 10.55 |
| 0.301 | 0.133 | 3.94 | 10.06 | 1.15 〃 | +0.42 | 10.48 |
| 0.321 | 0.112 | 4.12 | 9.83 | $7.59\times10^{-5}$ | +0.51 | 10.39 |
| 0.341 | 0.092 | 4.26 | 9.74 | 5.50 〃 | +0.62 | 10 36 |
| 0.361 | 0.071 | 4.43 | 9.57 | 3.72 〃 | +0.76 | 10.33 $pK'_{b_1}$ |
| 0.381 | 0.051 | 4.63 | 9.37 | 2.34 〃 | +0.93 | 10.30 |
| 0.401 | 0.030 | 4.89 | 9.11 | 1.29 〃 | +1.18 | 10.29 |
| 0.421 | 0.010 | 5.44 | | | | |
| 0.425 | 0.005 | 6.13 | | | | |
| 0.430 | 0.000 | 6.45 | | | | |
| 0.441 | 0.010 | 7.26 | | $C_{OH^-}$ | | |
| 0.461 | 0.031 | 7.99 | 6.01 | $9.77\times10^{-7}$ | *B* | |
| 0.481 | 0.051 | 8.53 | 5.47 | $3.39\times10^{-6}$ | +0.57 | 9.10 |
| 0.501 | 0.071 | 8.76 | 5.24 | 5.75 〃 | +0.33 | 9.14 |
| 0.521 | 0.092 | 8.95 | 5.05 | 8.91 〃 | +0.20 | 9.15 |
| 0.541 | 0.113 | 9.11 | 4.89 | $1.29\times10^{-5}$ | +0.05 | 9.16 |
| 0.562 | 0.135 | 9.26 | 4.74 | 1.82 〃 | −0.10 | 9.16 $pK'_a$ |
| 0.584 | 0.156 | 9.41 | 4.59 | 2.57 〃 | −0.26 | 9.15 |
| 0.604 | 0.177 | 9.53 | 4.42 | 3.80 〃 | −0.43 | 9.15 |
| 0.625 | 0.199 | 9.74 | 4 26 | 5.50 〃 | | |

* Blank for distilled water.

† $A = \log \dfrac{C_a - C_{A^-} + C_{H^+}}{C_{A^-} - C_{H^+}}$ †† $B = \log \dfrac{C_a - C_{B^+} + C_{OH^-}}{C_{B^+} - C_{OH^-}}$

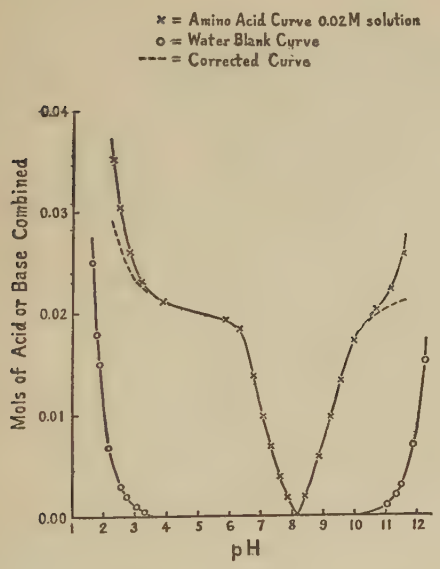



FIG. 2. Titration curves of canavanine

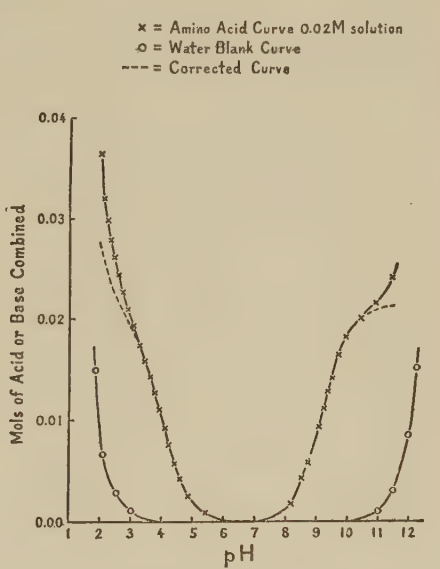



FIG. 3. Titration curves of canaline

On inspection of Figs. 2 and 3 and Table III, it is noticed that the basic group of canavanine has a basicity which is weaker than the guanidine group of arginine and a little stronger than the imidazole group of histidine. Besides the $\alpha$-amino group, canaline possesses a basic group which is so weak that it is a little difficult to distinguish it from $\alpha$-amino group. However, the apparent free energy changes due to ionization of amino acids show that the $pK'_{b_1}$ of canaline, whose $\Delta F'_{298}$ = 14,000 calories, does not correspond to the $\alpha$-amino group which generally has the value of $\Delta F'_{298}$ = 16,000 calories.[6] Therefore, $pK'_{b_1}$ is to be referred to the hydroxylamino group.

The result that canaline is a weaker basic compound than either ornithine or hydroxylamine seems to verify the formula which has been ascribed to canaline. Hydroxylamine possesses less basicity than ammonia, because one of the hydrogen atoms of ammonia is replaced by a negative group, i.e., hydroxyl group. In the same way, if the hydroxyl group of hydroxylamine is substituted by a more or less negative group like $-R-CH(NH_2)-COOH$, the resulting compound should be a less basic one than its mother substance. This is the case of canaline.

On the other hand, if it is supposed that canavanine possesses a guanidine

TABLE III

*Thermodynamic Data for Canavanine and Canaline at 25° and Comparisons of these Constants with Those of Arginine and Histidine.*

| Amino acids | pK′ | | pI | | $\Delta F'_{298}$ calories | Group responsible for pK′ |
|---|---|---|---|---|---|---|
| | | | exp. | cal. | | |
| Canavanine | $pK'_a$ | 9.25±0.03 | 8.10 | 7.93 | 12,600 | −COOH |
| | $pK'_{b_1}$ | 7.40±0.05 | | | 10,100 | $-O-NH-C\begin{matrix}\diagup NH_2\\ \diagdown NH\end{matrix}$ |
| | $pK'_{b_2}$ | 11.45±0.10 | | | 15,700 | $\alpha-NH_2$ |
| Canaline | $pK'_a$ | 9.15±0.05 | 6.45 | 6.46 | 12,500 | −CCOH |
| | $pK'_{b_1}$ | 10.30±0.05 | | | 14,000 | $-O-NH_2$ |
| | $pK'_{b_2}$ | 11.55±0.10 | | | 15,800 | $\alpha-NH_2$ |
| Arginine* | $pK'_a$ | 12.48 | | 10.76 | 17,000 | −COOH |
| | $pK'_{b_1}$ | 4.96 | | | 6,700 | $-NH-C\begin{matrix}\diagup NH_2\\ \diagdown NH\end{matrix}$ |
| | $pK'_{b_2}$ | 11.98 | | | 16,400 | $\alpha-NH_2$ |
| Histidine* | $pK'_a$ | 9.17 | | 7.59 | 12,500 | −CCOH |
| | $pK'_{b_1}$ | 8.00 | | | 10,900 | Imidazole |
| | $pK'_{b_2}$ | 12.18 | | | 16,600 | $\alpha-NH_2$ |

* C. L. A. SCHMIDT, W. K. APPLEMANN and PAUL L. KIRK: J. Biol. Chem., **81**, 723 (1929).

group, the fact that canavanine has a far smaller basic dissociation constant than arginine may be ascribed to the presence of the guanidoxy-oxygen atom rather than to the length of the carbon chain.

## Summary

1. The apparent dissociation constants and the corresponding free energy change for canavanine and canaline have heen determined at 25°.

2. It is shown that the basic group of canavanine is far weaker than the guanidine group of arginine and a little stronger than the imidazole group of histidine. The basic group of canaline other than the $\alpha$-amino group is so weak that it is a little difficult to distinguish it from the $\alpha$-amino group.

3. The isoelectric point of canavanine was obtained from the titration curve to be 8.10, while the calculated value from the data for $pK'_{b_1}$, $pK'_{b_2}$ and $pK'_a$ was 7.93. The isoelectric point of canaline was 6.45 from the titration curve and 6.46 calculated from the data for $pK'_{b_1}$, $pK'_{b_2}$ and $pK'_a$.

## CHAPTER II

### On the Nature of the Reaction which Takes Place between Certain Amino Acids and Formaldehyde

The present paper deals with the determination of the equilibrium constants of the reactions which take place between certain amino acids and formaldehyde, and with the postulation of the probable mechanism of the reaction. Since SCHIFF[7] showed that formaldehyde reacts with primary and secondary aliphatic amines, a number of papers on this subject have appeared. SÖRENSEN[8] applied the reaction to the determination of amino acids. HARRIS[9] studied the influence of formaldehyde on the dissociation constant of amino acids, and used the behavior of the amino acids in formaldehyde as a proof of the zwitter ion structure. LEVY[10] first worked out the equilibrium in the formol titration and concluded on the basis of a mathematical treatment of the data that two molecules of formaldehyde combine with one molecule of amino acid. On the other hand, a number of investigators[11] have isolated compounds from mixtures of amino acids or their salts and formaldehyde, and have found that the nature of the compounds depended on the conditions under which they were prepared.

It is very interesting to know whether amino acids are capable of uniting with two or three molecules of formaldehyde as is indicated by LEVY,[10] and BERGMANN,[11] or else only one as was postulated originally by SCHIFF.[7] As far as the present experiments go, one molecule of an amino acid (except a basic one) may combine with one molecule of formaldehyde in alkaline region. The mechanism of the reaction has been believed generally to involve the formation of a methylene compound by condensation. It has not yet been definitely shown, however, whether the reaction product is a methylene compound or an associated compound, the latter being assumed by LEVY.[10] It is postulated here that the reaction product most probably may be a molecular compound which is formed by

the residual charge of both components or by co-ordinate valency. The probable reasons for this hypothesis are discussed later on.

The equilibrium constant was calculated by using the amount of the molecular compound[1] which is formed at various concentrations of formaldehyde. The amount of the molecular compound is readily calculated, with the Henderson-Hasselbalch equation (7) and (8), by determining pH values before and after the addition of a certain amount of formaldehyde to the buffer system composed of an amino acid and its salt.

$$pH_1 = pK'_a + \log([A^-] / [{}^+A^-]) \quad (7)$$

$$pH_2 = pK'_a + \log([A^- - z^-] / [{}^+A^-])^* \quad (8)$$

where $A^-$ = the anion of the amino acid, ${}^+A^-$ = the zwitter ion of the amino acid, and $z^-$ = the molecular compound formed.

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[1] This term is used to designate the reaction product for the reason which will be discussed later.

* This formulation remains the same whether it is assumed that the anionic form (1) of the amino acid or the zwitter ionic form (2) reacts with formaldehyde, because the $H^+$ of the $NH_3^+$ group is given off when $NH_3^+$ reacts with formaldehyde, and this $H^+$ is neutralized by the anion in the system which is now dealt with, yielding the equivalent amount of zwitter ion. Thus:

(1) $$R\begin{smallmatrix}NH_2\\COO^-\end{smallmatrix} + CH_2O \rightleftarrows R\begin{smallmatrix}\overset{+}{N}H_2CH_2\bar{O}\\COO^-\end{smallmatrix}$$

(2) $$R\begin{smallmatrix}NH_3^+\\COO^-\end{smallmatrix} + CH_2O \rightleftarrows R\begin{smallmatrix}\overset{+}{N}H_2CH_2\bar{O}\\COO^-\end{smallmatrix} + H^+$$

$$R\begin{smallmatrix}NH_2\\COO^-\end{smallmatrix} + H^+ \rightleftarrows R\begin{smallmatrix}NH_3^+\\COO^-\end{smallmatrix}$$

$$pH_2 = pK'_a + \log([A^- - z^-] / [{}^+A^- - z^- + z^-])$$

As is shown later, however, it is more probable that the anionic form combines with formaldehyde rather than the zwitter ionic form.

## Method

It has been noticed that when the pH of a solution containing formaldehyde is measured by the hydrogen electrode, a considerable time is required to reach the equilibrium potential. A glass electrode which had an accuracy of pH 0.01 was used. The potential was measured by means of a De Eds glass electrode voltmeter[4] which was connected with a galvanometer having a sensitivity of $10^{-8}$ ampere per mm. The potential of the electrode was standardized daily by means of a standard acetate buffer. The electrode behaved theoretically up to pH 9.8. The diffusion potential was reduced very much by making a clear cut liquid junction between the solution under test and the saturated potassium chloride. It is to be noticed that if the electrical connection were made through the capillary of the stop-cock, the potential would not be reproducible and it would lead to erroneous results. All of the experiments were carried out at 25°.

The formaldehyde used was Merck's acid-free product. The concentration of formaldehyde solution was determined by Romijn's method.[12] The samples of amino acids were commercial products which were purified by several recrystallizations.

## Equilibrium Constant of Reaction

The amount of the molecular compound which was formed, $z^-$ was calculated by applying the principle aforementioned. The equilibrium constant was calculated by substituting the experimental values of $z^-$ in the following formula.

$$A^- + nF \rightleftarrows AF_n^-$$
$$K_{e(n)} = [z^-] / ([A^- - z^-][F - nz^-]^n) \qquad (9)$$

The $K_{e(n)}$ values for glycine, alanine, and proline were calculated. In all cases, constant values were obtained only when one molecule of the amino acid is considered to react with one molecule of formaldehyde. The results are summarized in Table IV.

By knowing the equilibrium constant and using the following equation (10), the pH change in the alkaline region of the amino acid buffer on the addition of various amounts of formaldehyde can be calculated and, furthermore, the end-point of the titration in the presence of a certain amount of formaldehyde can

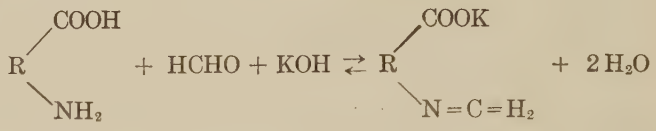
also be predicated.[2]

$$\mathrm{pH} = -\log K_e + \mathrm{pK'}_a + \log([z^-] / [{}^+A^-]) - \log[F - z^-] \qquad (10)$$

where $K_e$ = the equilibrium constant, $K'_a$ = the classical apparent acid dissociation constant, and $[F]$ = the concentration of formaldehyde in moles.

## Discussion

SÖRENSEN[3] supposed that the reaction involved in the formol titration might be as follows:



This hypothesis was based on the reaction product which SCHIFF had isolated and is generally adopted in many text-books. The reaction product in question was isolated by a number of workers besides SCHIFF. The isolated compounds seemed to be variable, depending on the conditions for their preparations. BERGMANN[11] isolated a triformyl compound of glycine from neutral solution, and observed the transformation of the triformyl into monoformyl compound by the addition of baryta. In connection with these facts, the polymerization and depolymerization of formaldehyde seem to play an important rôle. In acidic and neutral solutions, formaldehyde exists mostly in the polymerized form,[13] but is depolymerized by changing to an alkaline reaction. JAHODA[14] has shown recently that the formaldehyde is depolymerized with the increase of hydroxyl ion and with the rise of temperature.

---

[2] The calculated pH values at the end-point of the formol titration in 10 per cent formaldehyde are as follows: glycine 8.49, alanine 9.50 and proline 9.25, respectively. The derivation of the equation is as follows:

$$K_e = [z^-] / ([A^- - z^-][F - z^-])$$

$$\log K_e = \log([z^-] / [A^- - z^-]) - \log[F - z^-]$$

$${}^+A^- + \mathrm{OH}^- \rightleftarrows A^- + \mathrm{H_2O}; \quad [A^-] = ([{}^+A^-][\mathrm{OH}^-]) / K_B$$

$$\log K_e = \log([z^-] K_B / [{}^+A^-]_e [\mathrm{OH}^-]_e) - \log[F - z^-]$$

$$-\log[\mathrm{H}^+]_e = -\log K_e + \log(K_B / K_w) + \log([z^-] / [{}^+A^-]_e) - \log[F - z^-]$$

$$\mathrm{pH} = -\log K_e + \mathrm{pK'}_a + \log([z^-] / [{}^+A^-]_e) - \log[F - z^-]$$

where $[\ ]_e$ = the equilibrium concentration, $K_B$ = the zwitter ion constant, $K'_a$ = the apparent classical dissociation constant, and $K_w$ = the dissociation constant of water.

TABLE IV

*Equilibrium Constants.*

| $F$ | pH | $z^-$ | $K_{e(1)}$ | $K_{e(2)}$ | $K_{e(1/2)}$ |
|---|---|---|---|---|---|
| | | | | $\times 10^{-2}$ | $\times 10^{-2}$ |
| | Glycine | $[A^-]/[{}^+A^-] = 0.050/0.047$ | | | |
| 0.0000 | 9.68 | 0.0000 | | | |
| 0.0370 | 9.25 | 0.0335 | 491 | | 12 |
| 0.0555 | 8.90 | 0.0443 | 454 | | 28 |
| 0.0647 | 8.73 | 0.0471 | 454 | | 39 |
| 0.0740 | 8.59 | 0.0487 | 452 | | 51 |
| 0.0832 | 8.47 | 0.0498 | 460 | | 64 |
| 0.0925 | 8.37 | 0.0504 | 466 | | 76 |
| 0.1018 | 8.28 | 0.0509 | 479 | | 88 |
| | | | $465 \pm 13$ | | |
| | Alanine | $[A^-]/[{}^+A^-] = 0.053/0.044$ | | | |
| 0.0000 | 9.81 | 0.0000 | | | |
| 0.0370 | 9.63 | 0.0238 | 61.7 | | 5.6 |
| 0.0555 | 9.52 | 0.0304 | 53.7 | | 7.4 |
| 0.0740 | 9.42 | 0.0351 | 50.4 | 1355.0 | 9.5 |
| 0.0925 | 9.34 | 0.0380 | 46.5 | 93.0 | 11.5 |
| 0.1110 | 9.26 | 0.0406 | 46.5 | 36.9 | 14.6 |
| 0.1295 | 9.19 | 0.0425 | 46.5 | 20.9 | 17.7 |
| 0.1480 | 9.12 | 0.0441 | 47.0 | 13.8 | 22.0 |
| 0.1665 | 9.05 | 0.0453 | 48.5 | 9.8 | 26.5 |
| | | | $50.1 \pm 4.9$ | | |
| | Proline | $[A^-]/[{}^+A^-] = 0.050/0.050$ | | | |
| 0.0000 | 10.58 | 0.0000 | | | |
| 0.0800 | 9.98 | 0.0374 | 69.7 | 1090.0 | 19.2 |
| 0.1200 | 9.78 | 0.0421 | 68.4 | 41.6 | 33.2 |
| 0.1600 | 9.65 | 0.0442 | 65.8 | 14.9 | 47.6 |
| 0.2000 | 9.54 | 0.0455 | 65.4 | 9.4 | 63.6 |
| | | | $67.3 \pm 3.4$ | | |

The reaction system $= 10\,cc$ of $0.2\,M$ amino acid $+ x\,cc$ of 1 per cent formaldehyde $+ (10 - x)\,cc$ of water.

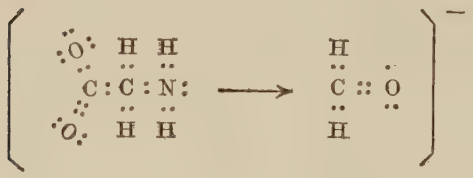
$F$ = the concentration of formaldehyde in moles, $z^-$ = the molecular compound formed, $A^-$ = the anionic form of amino acids, ${}^+A^-$ = the zwitter ionic form of amino acids.

$K_{e(1)}$ = equilibrium constant for the reaction $A^- + F \rightleftarrows AF^-$

$K_{e(2)}$ = equilibrium constant for the reaction $A^- + 2F \rightleftarrows AF_2^-$

$K_{e(1/2)}$ = equilibrium constant for the reaction $2A^- + F \rightleftarrows A_2F^-$

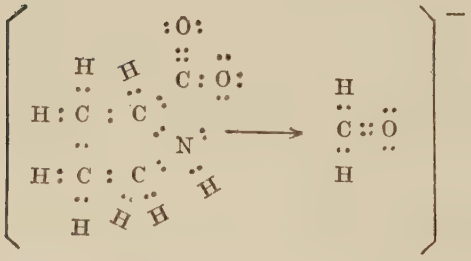
The calculation of the equilibrium constant,[3] reported in the present paper, shows that one molecule of amino acid combines with one molecule of formaldehyde from pH 10.0 to 8. This is also true in the case of *l*-proline. According to SÖRENSEN'S hypothesis, however, the condensation compound, $(C_5H_7O_2NK)_2{=}CH_2$, is expected to result. It is rather reasonable, therefore, to presume that the removal of hydrogen by condensation with formaldehyde may not be involved necessarily in the reaction. The mode of reaction between amino acids and formaldehyde which does not involve the condensation between two components will be clearly understood by considering the modern electronic theory of valency. A formaldehyde molecule exists as the dipolar molecule, i.e., $\overset{+}{C}H_2\,\overset{-}{O}$, since a formaldehyde molecule has a very large dipole moment. On the other hand, the amino or imino group of the anionic form of amino acid has two unshared electrons. If formaldehyde is added, therefore, to an amino acid solution, two components are expected to react by a co-ordinate valency, the lack of electron in the carbon atom of formaldehyde molecule being supplied by two unshared electrons of the amino group as illustrated in the following formula.[4]



or

$\overset{-}{O}OC-CH_2-\overset{+}{N}H_2-\overset{-}{C}H_2O$

Glycine-Formaldehyde



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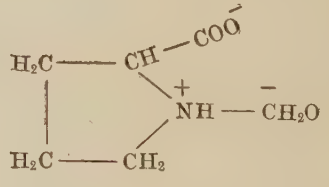

Proline-Formaldehyde

[3] It was assumed that formaldehyde might exist in the depolymerized form around pH 9, hence the analytical concentration of formaldehyde was used for the calculation of the equilibrium constant.

[4] This type of expression for the co-ordination compound has been adopted according to Sidgwick.[15]

It follows that an $\overset{+}{N}H_3$ group in which the negative residual charge of $-NH_2$ is changed to a positive one has less tendency to form the molecular compound.

If neutral formaldehyde is added to a neutral solution of amino acid or its ester, a molecular compound should form between polymerized formaldehyde and the amino acid or its ester.[5] This is why BERGMANN[11] was able to isolate a triformyl compound. This also explains why LEVY[10] obtained results which showed the formation of a diformyl compound. By the addition of a large amount of formaldehyde, the pH of the half neutralized amino acid was lowered to pH 4, under which conditions aldehyde exists in a polymerized form. It may not be safe to draw any conclusion from experiments which deal with high concentrations of formaldehyde where the accuracy of the determination of the pH by means of the hydrogen electrode is quite doubtful and the dielectric constant of the solution may be changed a great deal. By careful inspection of LEVY's data, it will be noticed that in the pH range above 8 the monoformyl compound seems to predominate. The foregoing mechanism of the reaction is to be regarded for the present as a hypothesis which fits the known facts.

Another type of mechanism which should be taken into consideration is the neutralization of free amino groups by means of the very weak acidic group of formaldehyde. Formaldehyde combines with sodium hydroxide at a high pH. The $pK'_a$ value of formaldehyde was determined recently to be 12.9 by LEVY.[10] There may be one objection to this idea; viz., at pH 9, or less, when formaldehyde is able to react with amino groups, the ionization of formaldehyde as an acid does not take place and so there is the least possibility for neutralizing the amino group.

A polarographic study with the mercury dropping cathode[16] was also made to elucidate the mode of combination between amino acid and formaldehyde. The results showed that, in alkaline solution, the diffusion current of formaldehyde which corresponds to the concentration of formaldehyde did not change whether or not glycine was added. When the glycine is added to formaldehyde, almost all of the formaldehyde is in combination with glycine as is seen from the value of the equilibrium constant. If formaldehyde should condense with the glycine, forming the methylene compound, it might be expected that the observed reduction potential should be appreciably different from that of formaldehyde. This

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[5] The polyformyl compound may be formulated as follows:

$$\overset{-}{O}OC-CH_2-\overset{+}{N}H_2-CH_2O-CH_2O-CH_2\overset{-}{O}.$$

was not the case, so the conclusion will be drawn that formaldehyde combines with the amino acid without the substantial change in its constitution.

The value of the equilibrium constant for glycine is almost nine times as great as that for alanine. Glycine has, therefore, a strong affinity for formaldehyde.

HARRIS[9] explained the shift of the titration curve of the amino acid which takes place by the addition of formaldehyde by assuming that the $K'_a$ value of the amino acid increases 1000-fold. The equilibrium constant of the reaction, however, was entirely neglected. The shift of the titration curve and each point on the titration curve in the alkaline region may be calculated from equation 10. The shift of the curve is characterized by the amount of formaldehyde and the equilibrium constant. The formol titration curve does not deal with the free methylene fatty acid and its salt, but with a free amino acid and the sodium or barium salt of the molecular compound which is formed during the titration. The inflexion point of the formol titration curve does not show the $pK'_a$ value of the methylene fatty acid. It is to be emphasized, accordingly, that the course of the formol titration curve is characteristic of the formation of the addition compound.

**Summary**

1. The calculation of the equilibrium constant of the reaction which takes place between certain amino acids and formaldehyde shows that one molecule of amino acid combines with one molecule of formaldehyde from pH 10 to 8. The values for glycine, alanine, and proline are $465 \pm 13$, $50.1 \pm 4.9$, and $67.3 \pm 3.4$, respectively.

2. The mechanism of the reaction in question is discussed. The reaction product most probably may be a molecular compound and not a methylene compound.

## CHAPTER III

## The Solubilities of *l*-Proline and *l*-Hydroxyproline in Water, the Calculated Heats of Solution, and the Apparent Molal Volume of *l*-Hydroxyproline

The data of the solubility of amino acid and of the effect of temperature on its solubility are not only useful to the isolation of amino acid but also important as a criterion for the purity of amino acid. The solubility data at various temper-

atures enable us to postulate about the physico-chemical form of amino acid in solution and also to calculate the heat of solution of the compound. DALTON and SCHMIDT[17] have obtained from the solubility data certain interesting results which show that *dl*-alanine, *dl*-leucine, *dl*-aspartic acid are racemic compounds while *dl*-glutamic acid is a racemic mixture.

The solubility data of many amino acids have been obtained recently by DALTON and SCHMIDT[17] and by DUNN, ROSS, and READ.[18] There have been left, however, certain amino acids which solubilities have not yet been determined, because of the fact that these solubilities are so high that it requires a large amount of pure amino acid to carry out the determination. Accordingly, attempts have been made to carry out the solubility determination on a micro scale. Several methods for the determination of weight and volume of a small quantity of solution[19] [20] have been examined, but any desirable accuracy has not been obtained. Hence, a new micro technique has been developed. The solubility determinations of *l*-proline and *l*-hydroxyproline have been carried out by a micro technique, since no precise determinations appear to have been attempted. Due to the high solubility of *l*-proline and *l*-hydroxyproline it was not feasible to carry out solubility determinations over as great a temperature range as in the previously reported studies.[17] There is a tendency for these amino acids, especially hydroxyproline, to darken at higher temperatures, hence the determination was carried out in oxygen-free atmosphere. The solubility of *l*-proline was determined at five different temperatures over the range of $10.2 \sim 51°$, and of *l*-hydroxyproline was determined at eight different temperatures over the range of $0 \sim 64°$. From these solubility data have been obtained solubility equations which show the relation between the solubilities and the temperature. Furthermore, the heat of solution of these amino acids has been calculated from the solubility equation. The apparent molal volume for *l*-hydroxyproline has also been calculated from the determination of density of the saturated solution and the value was compared with the TRAUBE'S empirical value with a fairly close agreement.

## Experimental

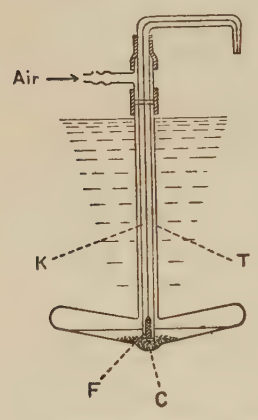
(1) *The Preparation of Amino Acids.*

*l*-Proline used was a commercial product (Hoffmann-La Roche). *l*-Hydroxyproline was isolated from gelatine according to KLABUNDE method.[21] The isolated

amino acid was at the same time compared with a commercial product (Hoffmann-La Roche). The sample of *l*-proline was obtained by recrystallizing several times from a mixture of alcohol and ether until its solubility became a constant value. The sample of *l*-hydroxyproline was recrystallized three times from alcohol before its solubility became constant.

(2) *The Method for the Determination of Solubility.*

The apparatus for the determination was similar to that which was used by DALTON and SCHMIDT.[17] The size of the apparatus was made as small as possible. Fig. 4 shows a set of apparatus for the determination. The saturated solution of the amino acids was made in a following way. Into a T-tube (Fig. 4) about 2 cc conductivity water were poured and boiled to remove carbon dioxide as well as to sterilize the vessel. After cooling, an appropriate amount of the sample was added and the tube was stoppered. The tube was then dipped into an oil thermostat and shaken to and fro by a mechanical device driven by a motor until the amount of the solute became constant at different time-periods. The two days' shaking was found ordinarily enough to attain the equilibrated saturated solution. At every determination, care was taken as to the state of saturation, namely, the determination was carried out by permitting a supersaturated solution and an undersaturated solution, in the presence of an excess of the particular amino acid, to come to equilibrium at the desired temperature. In the case of *l*-hydroxyprolire, a difficulty arose in the determination at higher temperatures since the compound seems to decompose above 40°, taking into account the fact that the solution became brownish within a 24-hour period. Consequently, in making the saturated solution of *l*-hydroxyproline above 40°, was paid such a special precaution as to fill up the tube with oxygen-free air which gave the promising result.




T: T-tube for making the saturated solution.
K: Thick-walled capillary tube.
F: Filter of cotton.
C: Sample amino acid.

FIG. 4

In order to take a sample of the saturated solution, a thick walled capillary tube shown in Fig. 4 was used. The saturated solution was forced through a filter of cotton and taken into a small bottle by applying air pressure under 150 mm of

mercury instead of taking a sample by suction. In this way evaporation losses were kept at a minimum, and as the filtration was rapid, there was little or no crystallization before the solution reached the bottle. The saturated solution was taken in a micro pipette of about 100 c.mm. capacity shown in Fig. 5 which enables the measurements of density and volume of the solution. The micro pipette possessed a uniform capillary stem whose diameter was about 0.3 mm; 1 mm of the capillary corresponded to about 0.07 c.mm. The volume of the pipette was calibrated with the aid of mercury. The volume of amino acid solution which was contained in the pipette was estimated by measuring the length on the stem which was not filled with the solution, since the volume occupied by the solution can be calculated by knowing $x$ in the following formula:

Fig. 5

$$v = V + (a - x) \times k \qquad (11)$$

where $v$ = volume to be estimated, $V$ = volume of mercury which occupied the pipette under calibration up to a certain point on the stem A, $a$ mm apart from the end of A, $x$ = length to be measured on the stem A which was not filled with the solution, $k$ = volume corresponding to 1 mm of the capillary of the stem A. For the estimation of density, the pipette was filled with the solution to a certain point on the stem A, and the length which was not filled with the solution was measured. After the tip of the pipette was wiped with wet cotton, the pipette was left in the balance room at 25° for 15 to 30 minutes, after which it was weighed on a Kuhlmann micro balance. The capillary stem and the tip of the pipette were so narrow that almost no loss of solution by evaporation was observed after standing in the balance room for a 2-hour period. The amino acid content of the solution, after thoroughly rinsing the pipette, was estimated on the basis of the nitrogen content. For this purpose the micro Kjeldahl method of Parnas and Wagner[22] was used.

## Results and Discussion

The solubility data were expressed in number of mols of solute per 1000 grams of water since the values are independent of the temperature. Fourteen solubility estimations of *l*-proline and thirty three estimations of *l*-hydroxyproline were

carried out, and the data are given in Tables V and VI.

The relationships between the logarithms of mol fraction of the solute and the temperature have been shown to be a linear function as represented graphically in Fig. 6. The equations which represent these relationships have been developed by using the method of least squares. For the calculation of heats of solution of the amino acids, use was made of the well-known van't Hoff equation as modified by SCHRÖDER[23] which may be written as follows:

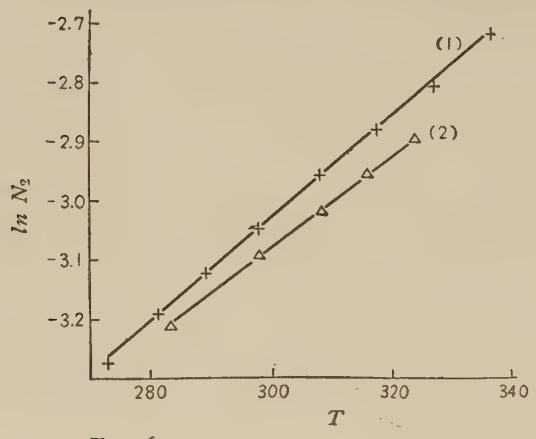



FIG. 6. Solubility of *l*-hydroxyproline (1) and *l*-proline (2). In the case of *l*-proline, the values of the ordinates are 1.5 less than the values indicated in the figure.

$$\partial \ln N_2 / \partial T = \Delta H / RT^2 \qquad (12)$$

$N_2$ = mol fraction of the solute

$T$ = absolute temperature

$R$ = gas constant

$\Delta H$ = differential heat of solution (calories per mol).

The solubility equations are applicable only to the temperature ranges in which solubility estimations were carried out. Regarding the heat of solution of *l*-proline and *l*-hydroxyproline, ZITTLE and SCHMIDT[24] measured it by using an accurate calorimeter. Their values were $> 300 \pm 50$ calories for 8 mol solution of *l*-proline and $> 1500 \pm 75$ calories for 2 mol solution of *l*-hydroxyproline, respectively. Since those values involve fairly great error, the present values for $\Delta H$ are perhaps more nearly correct than those reported by them. The coefficients of the equations which represent these relationships, together with the calculated values for $\Delta H$, the heat of solution, are given in Table VII.

According to the theory underlying the fundamental relationship between the mol fraction of the solute $N_2$ and the absolute temperature $T$, the melting point of the compounds should be that temperature at which $N_2 = 1$. Assuming that

Table V

*Solubility Data of l-Proline.*

| Temperature (°T) | Mode of saturation | Weight of sample (mg) | Weight of proline in sample (mg) | Solubility $S$ exp. (g / 1000 g solv.) | Solubility $S$ calc. (g / 1000 g solv.) | log $S$ | $m$ (mol / 1000 g solv.) | $N_2 \times 10^2$ | 1n $N_2$ |
|---|---|---|---|---|---|---|---|---|---|
| 283.3 | SS | 87.560 | 51.231 | 1410 | 1406 | 3.1492 | 12.25 | 0.1807 | −1.7089 |
| | SS | 92.680 | 53.978 | 1395 | | 3.1446 | 12.12 | 0.1792 | −1.7170 |
| 298.1 | US | 88.950 | 55.172 | 1632 | | 3.2127 | 14.18 | 0.2034 | −1.5929 |
| | US | 93.840 | 57.964 | 1615 | | 3.2082 | 14.04 | 0.2020 | −1.5998 |
| | US | 89.360 | 55.172 | 1620 | 1623 | 3.2093 | 14.07 | 0.2022 | −1.5988 |
| | US | 93.310 | 57.718 | 1622 | | 3.2101 | 14.09 | 0.2024 | −1.5978 |
| | SS | 89.770 | 56.075 | 1664 | | 3.2216 | 14.46 | 0.2066 | −1.5773 |
| 308.6 | US | 89.240 | 57.389 | 1802 | | 3.2553 | 15.65 | 0.2201 | −1.5140 |
| | US | 93.660 | 59.930 | 1777 | 1769 | 3.2497 | 15.44 | 0.2176 | −1.5254 |
| | SS | 90.235 | 57.964 | 1796 | | 3.2543 | 15.60 | 0.2194 | −1.5172 |
| | SS | 94.840 | 60.509 | 1763 | | 3.2463 | 15.32 | 0.2163 | −1.5313 |
| 316.1 | US | 90.160 | 59.523 | 1943 | 1931 | 3.2885 | 16.88 | 0.2332 | −1.4562 |
| 324.1 | US | 88.925 | 59.277 | 2069 | 2687 | 3.3158 | 17.97 | 0.2446 | −1.4082 |
| | US | 94.615 | 64.253 | 2116 | | 3.3255 | 18.38 | 0.2487 | −1.3917 |

SS = the equilibrium for saturation was made from a supersaturated condition.

US = the equilibrium for saturation was made from an undersaturated condition.

$S = w / (W - w) \times 1000$; W = weight of a sample taken for the estimation, $w$ = weight of the solute dissolved in the sample taken.

$m$ = molality.

$N_2$ = mol fraction of the solute $= n_2 / (n_1 + n_2)$; $n_1$ = no. of mols of water in 1000 g water, $n_2$ = no. of mols of the solute dissolved in 1000 g water.

TABLE VI

*Solubility Data of l-Hydroxyproline.*

| Temperature (°T) | Mode of saturation | Weight of sample (mg) | Volume of sample 25°C ($mm^3$) | Absolute density | Weight of OH-proline in sample (mg) | Solubility $S$ exp. | Solubility $S$ cal. | log $S$ | $m$ | $N_2 \times 10^2$ | 1n $N_2$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | $\frac{g}{1000\,g\ solv.}$ | | | | | |
| 273.1 | SS | 81.985 | 75.880 | 1.0804 | 18.187 | 285.1 | 288.6 | 2.4550 | 2.175 | 3.770 | −3.2782 |
| | SS | 85.490 | 79.054 | 1.0813 | 18.934 | 284.5 | | 2.4541 | 2.170 | 3.762 | −3.2803 |
| | SS* | 81.825 | 75.711 | 1.0807 | 18.243 | 286.9 | | 2.4577 | 2.188 | 3.792 | −3.2722 |
| | SS | 85.775 | 79.364 | 1.0808 | 19.069 | 285.9 | | 2.4562 | 2.181 | 3.780 | −3.2754 |
| 281.3 | SS | 82.450 | 75.877 | 1.0866 | 19.645 | 312.8 | 310.3 | 2.4953 | 2.386 | 4.121 | −3.1891 |
| | SS | 86.610 | 79.703 | 1.0867 | 20.523 | 310.6 | | 2.4922 | 2.370 | 4.095 | −3.1955 |
| | SS* | 82.255 | 75.668 | 1.0870 | 19.626 | 313.3 | | 2.4860 | 2.390 | 4.127 | −3.1877 |
| | SS* | 86.140 | 79.232 | 1.0871 | 20.430 | 311.0 | | 2.4928 | 2.373 | 4.100 | −3.1942 |
| 289.3 | US | 82.850 | 75.837 | 1.0627 | 20.747 | 334.0 | 333.7 | 2.5238 | 2.548 | 4.389 | −3.1260 |
| | US* | 86.640 | 79.265 | 1.0932 | 21.794 | 336.0 | | 2.5263 | 2.563 | 4.413 | −3.1207 |
| | SS | 82.590 | 75.541 | 1.0934 | 20.766 | 335.9 | | 2.5262 | 2.563 | 4.413 | −3.1207 |
| | SS | 86.890 | 79.474 | 1.0935 | 21.776 | 334.4 | | 2.5243 | 2.551 | 4.394 | −3.1249 |
| 298.1 | US | 83.355 | 75.795 | 1.0997 | 22.205 | 363.1 | 361.1 | 2.5600 | 2.770 | 4.753 | −3.0463 |
| | US* | 87.140 | 79.232 | 1.0998 | 23.178 | 362.4 | | 2.5592 | 2.765 | 4.753 | −3.0482 |
| | SS | 83.370 | 75.813 | 1.0997 | 22.205 | 363.0 | | 2.5599 | 2.769 | 4.751 | −3.0468 |
| | SS | 87.185 | 79.265 | 1.0999 | 23.121 | 360.9 | | 2.5574 | 2.753 | 4.725 | −3.0523 |
| 308.4 | US | 83.785 | 75.727 | 1.1065 | 23.850 | 397.9 | 396.0 | 2.5998 | 3.035 | 5.184 | −2.9595 |
| | US* | 87.650 | 79.180 | 1.1070 | 24.916 | 397.1 | | 2.5989 | 3.029 | 5.174 | −2.9616 |
| | SS | 83.910 | 75.800 | 1.1067 | 24.000 | 400.0 | | 2.6021 | 3.051 | 5.210 | −2.9547 |
| | SS | 89.900 | 81.900 | 1.1072 | 24.972 | 397.0 | | 2.5988 | 3.029 | 5.174 | −2.9616 |

(*Continued*)

TABLE VI

(*Continued*)

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 317.9 | US | 83.640 | 75.101 | 1.1141 | 25.233 | 432.1 | | 2.6356 | 3.294 | 5.601 | −2.8822 |
| | US | 83.550 | 74.907 | 1.1153 | 25.196 | 431.8 | | 2.6353 | 3.293 | 5.600 | −2.8824 |
| | US* | 87.880 | 78.760 | 1.1150 | 26.355 | 428.4 | 431.2 | 2.6319 | 3.268 | 5.560 | −2.8895 |
| | SS | 83.810 | 75.161 | 1.1151 | 25.383 | 434.5 | | 2.6380 | 3.314 | 5.634 | −2.8764 |
| | SS | 88.280 | 79.166 | 1.1152 | 26.636 | 432.1 | | 2.6356 | 3.294 | 5.601 | −2.8822 |
| 327.3 | US | 84.025 | 74.907 | 1.1220 | 26.822 | 468.9 | | 2.6711 | 3.577 | 6.054 | −2.8046 |
| | US | 87.895 | 78.315 | 1.1222 | 28.037 | 468.4 | 469.1 | 2.6707 | 3.573 | 6.047 | −2.8057 |
| | US* | 83.450 | 74.400 | 1.1216 | 26.163 | 462.0 | | 2.6646 | 3.525 | 5.971 | −2.8184 |
| | US* | 87.505 | 78.034 | 1.1214 | 27.757 | 464.6 | | 2.6671 | 3.544 | 6.000 | −2.8129 |
| 337.1 | US | 83.100 | 73.681 | 1.1280 | 28.112 | 511.2 | | 2.7086 | 3.900 | 6.564 | −2.7235 |
| | US | 87.555 | 77.576 | 1.1287 | 29.607 | 510.9 | 512.1 | 2.7083 | 3.897 | 6.560 | −2.7242 |
| | US* | 83.305 | 73.808 | 1.1287 | 28.206 | 511.9 | | 2.7091 | 3.905 | 6.572 | −2.7224 |
| | US* | 83.360 | 78.315 | 1.1283 | 29.981 | 513.5 | | 2.7105 | 3.917 | 6.591 | −2.7196 |

* Solubility estimations made on *l*-hydroxyproline which has been isolated by KLABUNDE method.

TABLE VII

*Coefficients of Solubility Equations* of l-Proline and l-Hydroxyproline, and their Heat of Solution.*

| Amino acid | $a_1$ | $b_1\times10^2$ | $a_2$ | $a_3$ | $b_3\times10^2$ | $a_4$ | $b_4\times10^2$ | Maximum dev.† | Stand. dev. | $\Delta H_{298}$ calculated |
|---|---|---|---|---|---|---|---|---|---|---|
| *l*-Proline | 3.1050 | 0.4206 | 1.0441 | −0.2407 | 0.2686 | −3.8586 | 0.7586 | +2.53% | +1.04% | 1340 |
| *l*-Hydroxy-proline | 2.4603 | 0.3891 | 0.3423 | −1.6575 | 0.8959 | −5.5906 | 0.8514 | −1.53% | +0.61% | 1506 |

* Solubility equations: $\log S = a_1 + b_1 t$ $\quad \ln m = a_3 + b_3 T$

$\log m = a_2 + b_1 t$ $\quad \ln N_2 = a_4 + b_4 T$

† Maximum deviation of the observed from the calculated values.

the appropriate equations obtained from the experimental data also holds for higher temperatures, the melting points of *l*-proline and *l*-hydroxyproline computed from the equations proved to be approximately 236° and 384°, respectively. As the decomposition points recorded by KOSSEL and DAKIN[25] for *l*-proline and by FISCHER[26] for *l*-hydroxyproline were 220 ~ 222° and 270°, respectively, it is considered probable that decomposition occurred before the melting point was reached.

Due to the tendency of *l*-proline to crystallize out of a saturated solution, it was not feasible to carry out density determinations. The density data of *l*-hydroxyproline at 25° only are reported. These data which are given in Table VI have enabled us to compute the apparent molal volumes of the amino acids by using the following equation[27]:

$$\varphi = (V - n_1 v_1) / n_2$$

$\varphi$ = apparent volume occupied by 1 mol of the solute
$V$ = total volume of the solution
$n_1$ = number of mols of the solvent in 1000 g solvent
$v_1$ = apparent volume occupied by 1 mol of the solvent
$n_2$ = number of mols of the solute dissolved in 1000 g of the solvent.

The results of the calculation are given in Table VIII. The average for the apparent molal volume of *l*-hydroxyproline is 85.62 ± 0.12 cc. Now, on the basis of the apparent molal volumes calculated from the empirical atomic volumes given by TRAUBE,[28] the calculated molal volume of *l*-hydroxyproline is 85.2 cc as will be seen from the following calculation:

$$N = 1.5 \quad C_5 = 49.5 \quad H_9 = 27.9 \quad \text{Carboxyl-0} = 5.5 \quad (\text{Hydroxyl-0})_2 = 0.8$$
$$C_4 H_9 CO (OH)_2 N = 85.2$$

The validity of the calculation is based on TRAUBE'S statement, "in den übrigen stickstoffhaltigen Ringen, wie Pyrrol, Pyrrolidin, Pyrazol, Trimethylenimid usw. sind die Volumendekremente und Spannungen nicht gross." Consequently, it is probable that the good agreement between the present value and the calculated value according to TRAUBE implies that the pyrrolidin ring of *l*-hydroxyproline may exists in normal condition without "Volumdekremente" and "Spannungen."

TABLE VIII

*Apparent Molal Volume of l-Hydroxyproline.*

| Temperature for saturation (°T) | $V$ | $\varphi$ | Temperature for saturation (°T) | $V$ | $\varphi$ |
|---|---|---|---|---|---|
| 273.1 | 1189.46 | 85.76 | 308.4 | 1262.63 | 85.57 |
| | 1187.92 | 85.24 | | 1262.06 | 85.55 |
| | 1190.80 | 85.86 | | 1265.02 | 85 93 |
| | 1189.77 | 85.66 | | 1261.74 | 85.44 |
| 281.3 | 1208.17 | 86.01 | 317.9 | 1285.43 | 85.76 |
| | 1206.03 | 85.69 | | 1283.78 | 85.28 |
| | 1208.19 | 85.83 | | 1281.08 | 85.11 |
| | 1205.96 | 85.56 | | 1284.18 | 85.37 |
| 289.3 | 1220.83 | 85.51 | 327.3 | 1309.18 | 85.61 |
| | 1222.10 | 85.51 | | 1308.50 | 85.52 |
| | 1221.78 | 85.38 | | 1303.50 | 85.27 |
| | 1220.30 | 85.21 | | 1306.05 | 85.53 |
| 298.1 | 1239.52 | 85.41 | 337.1 | 1339.71 | 86.35 |
| | 1238.77 | 85.29 | | 1338.62 | 86.42 |
| | 1239.43 | 85.41 | | 1339.50 | 86.19 |
| | 1237.29 | 85.13 | | 1341.40 | 86.41 |

## Summary

1. The solubilities of *l*-proline and *l*-hydroxyproline have been determined with the micro technique which has newly been developed.

2. The solubility-temperature relationships, within the temperature limits studied, can be shown by the following equations:

*l*-Proline $\log S = 3.1050 + 0.4206 \times 10^{-2} t$

*l*-Hydroxyproline $\log S = 2.4603 + 0.3891 \times 10^{-2} t$

3. The heats of solution of *l*-proline and *l*-hydroxyproline were calculated from the density determinations of the saturated solutions, and found to be $85.62 \pm 0.12$ cc. The reported value is in close agreement with that calculated from the empirical atomic volumes given by TRAUBE.

## CHAPTER IV

### On the Adsorption of Picrates of Diamino Acids by Charcoal

PHELPS and PETERS[29] studied the adsorption of histidine by Norit, and found that histidine was adsorbed with its maximum amount at pH 7. 3. The adsorption of several amino acids by charcoal has been systematically investigated by T. ITO.[30] He found that the surface tension of the solution of amino acid does not play such an important rôle in the adsorption, and that the amount adsorbed by charcoal depends upon kinds of amino acid as well as upon whether the amino acid exists in an ionized form or a zwitter ionic form.

However, taking into consideration that the adsorption phenomena deal with the chemical nature of the molecule of adsorbendum and adsorbent, a question will arise whether or not the adsorption of the ionized form of amino acid be influenced by the kinds of acid with which the amino acid forms a salt. No research has been made of the mechanism of adsorption of salt of basic amino acid; for example, which component of the salt, the cationic molecule of basic amino acid or anionic molecule of the acid component, orientates itself against the active surface of charcoal. To throw light on the mechanism of adsorption of amino acid by charcoal, an investigation has been made of the adsorption of the picrates of several diamino acids by charcoal. By determining the amount of each component which is adsorbed by charcoal, certain informations have been obtained of the probable mechanism of adsorption by charcoal. The adsorption of the diamino acid picrate, within the limits of the concentration studied, has been found to conform to FREUNDLICH'S adsorption isotherm. Comparisons are also made between the constants of the equation for each picrate.

#### Experimental

(1) *The preparation of charcoal-suspension.*

A product of Kokusan Kagaku Co. (extra pure) was used without purification. Instead of weighing a definite amouut of charcoal, an appropriate amount of its suspension in water was taken for each experiment. In order to prepare the suspension as homogeneously as possible, about 150 g of charcoal were well mixed

with 500 cc water, and the mixture was then poured into a 500 cc measuring cylinder. After standing for ten minutes, 350 cc of the upper layer of the suspension were taken, and left overnight. The part deposited was separated by decantation, and was suspended in about 350 cc of water. This suspension contained about 50 mg of charcoal per cc. For the adsorption experiment, use was made of the suspension which contains about 32 mg charcoal per cc.

(2) *The method of determining separately the adsorbed amount of the diamino acid cation and the picrate anion.*

The adsorption of the picrate by charcoal and the determination of adsorbed amount was carried out as follows. 25 cc of the solution, having the various concentrations of the picrate, are taken in an Erlenmeyer flask, 1 cc of the suspension of charcoal is added and leave it stand at 25° for 2 hours, the flask being shaken several times during the interval. After being filtered, the total nitrogen and amino nitrogen in the filtrate were determined by the micro KJELDAHL method and the micro VAN SLYKE method, respectively. In using the micro KJELDAHL method, the procedure was modified in such a way as to reduce the nitro-form nitrogen, before digesting the picrate, into amine by zinc dust and sulfuric acid. The molar concentration of amino acid was calculated from the value for amino nitrogen, and that of picric acid from the amount of nitrogen obtained by subtracting amino nitrogen from the total nitrogen. It is to be noted here that the filter paper did not adsorb amino acid cation nor picrate anion. This was confirmed by the preliminary experiment which showed that there was no difference between the amount of nitrogen of the filtrate and that of the supernatent solution obtained by centrifuge. The amount adsorbed by charcoal is the difference of the concentration of both components of picrate before and after shaking the solution with charcoal, and from this value, the amount adsorbed by each gram of charcoal may be obtained.

(3) *The purities of the picrates.*

The picrates used for the experiment were lysine monopicrate, arginine monopicrate, histidine dipicrate, canavanine dipicrate, and canaline dipicrate. The purities of these picrates have been determined by their total nitrogen- and amino nitrogen-content.

(a) LYSINE PICRATE $C_{12}H_{17}O_9N_5$

The amino nitrogen was determined by shaking the aqueous solution of the picrate with nitrous acid for one minute, leaving the mixture stand for thirty minutes, and further shaking for one minute (at about 20°).

| | |
|---|---|
| Calculated value of amino nitrogen | 0.2239 mg / cc solution |
| Experimental value " " " | 0.222 " " " |

Regarding the determination of the total nitrogen of lysine picrate, a difficulty arose in using the KJELDAHL method. HENDERSON,[31] KUTSCHER and STEUDEL,[32] and SÖRENSEN and ANDERSON[33] have showed that the determination of lysine nitrogen by the KJELDAHL method gives a smaller value which may be due to the formation of piperidine compound. Several experiments have been carried out to confirm this abnormal phenomenon. The following result shows that the digestion of lysine picrate takes a long time to get a constant value which is even lower than the theoretical value.

| Time-period for digestion* (h.) | Per cent of theoretical value |
|---|---|
| 1 | 93.30 |
| 3 | 94.43 |
| 6 | 96.10 |
| 10 | 96.50 |
| 13 | 97.73 |
| 15 | 97.63 |

Therefore, the digestion has always been carried out for fifteen hours. The following calculation, based on the results aforementioned, has been adopted for the determination of the picrate anion and lysine cation. Since the nitrogen content of lysine picrate can be determined to be as much as 97.6 % of the theoretical, and the picrate anion can be totally decomposed within one hour's digestion, the decomposition of lysine cation after the digestion for fifteen hours can be calculated and found to be 95.5 %. Accordingly, the amount of picrate anion was calculated from the difference between the total nitrogen and the amino nitrogen which was multiplied by 0.955.

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* 1 cc of 0.016 molar solution of lysine picrate was digested with 2 cc concentrated sulfuric acid together with 1 g of a mixture of potassium sulfate and copper sulfate (3 : 1).

(b) Arginine monopicrate $C_{12}H_{17}O_9N_7 \cdot 2H_2O$

The amino nitrogen was determined by shaking an aqueous solution of the picrate with nitrous acid for one minute, leaving for three minutes, and shaking further for one minute. Contrary to Sekiné's work,[34] the reaction for the additional 20 minutes did not increase the value for the amino nitrogen. The determination of the total nitrogen by the Kjeldahl method showed the purity to be 99.5 % of the theoretical. It was noticed that the Kjeldahl digestion for one hour is sufficient completely to decompose the picrate.

(c) Histidine dipicrate $C_{18}H_{15}O_{16}N_9 \cdot 2H_2O$

The determination of amino nitrogen and the total nitrogen was carried out in the same way as that of arginine picrate. These values show that the purity of the picrate is 99.5 %.

(d) Canavanine dipicrate $C_5H_{12}O_3N_4 \cdot 2C_6H_3N_3O_7$

Kitagawa and Yamada[3] reported that canavanine gives off half of its total nitrogen by the action of nitrous acid for two hours. However, the following experimental results show that the deamination of canavanine gradually takes place during three hours, giving off nitrogen as much as three quarters of its total nitrogen.

| Time-interval for the reaction between canavanine and nitrous acid | Temp. and atmos. press. | Nitrogen liberated expressed in % of total nitrogen |
|---|---|---|
| 5 min. | 23°, 767.2 mm | 50.50 |
| 30 min. | 20°, 769.3 〃 | 56.62 |
| 1 h. 20 min. | 23°, 766.7 〃 | 66.16 |
| 2 h. 30 min. | 21°, 765.8 〃 | 74.55 |
| 5 h. | 21°, 768.0 〃 | 74.55 |

For the calculation of canavanine, accordingly, the value for amino nitrogen obtained by the above procedure has been adopted. From the determination of the amino nitrogen and the total nitrogen, it has been shown that the purity of the sample picrate is 99.5 %.

(e) Canaline dipicrate $C_4H_{10}O_3N_2 \cdot 2C_6H_3N_3O_7$

The procedure for determining amino nitrogen by the van Slyke method was

the same as was used in arginine picrate. This gave an abnormal value which corresponded to $116 \pm 1\%$ of the theoretical value for $\alpha$-amino nitrogen. The following example shows that further reaction with nitrous acid for three hours gives the same value.

Sample: 0.004285 mol canaline picrate which contains 0.0600 mg $\alpha$-amino nitrogen per each cc.

| Time-interval for the reaction | Temp. and atmos. press. | Nitrogen liberated | |
|---|---|---|---|
| 3 min. | 23°, 770 mm | 0.07034 mg | 117 % of theor. |
| 1 h. 30 min. | 22°, 771 mm | 0.07034 mg | 〃 |
| 2 h. 30 min. | 22°, 770 mm | 0.07024 mg | 〃 |

Using the free canaline instead of its picrate, similar data were obtained. The reason why canaline gives off an excess of amino nitrogen is not clear. In calculating the amount of picrate anion, care was taken to subtract from the total nitrogen the amino nitrogen which is divided by 1.16. The determination of total nitrogen was carried out as usual, and showed that the purity of the picrate was 100.0 %.

## Results and Discussions

The adsorbed amount of each amino acid cation and picrate anion was determined by the same procedure as was adopted for determining the purity of each. The adsorbed amount is expressed in millimol of each component per gram of charcoal. The relation between the adsorbed amount and the equilibrium concentration is expressed by FREUNDLICH's adsorption isotherm. The experimental values are treated by the method of least square and the most probable values for the constants of the equation are obtained.

The results are given in Tables IX, X, XI, XII, and XIII. Figs. 7 and 8 clearly show the behavior of cation as well as anion of these picrates in the adsorption phenomena. As a reference to the picrate of the diamino acid, the adsorption of sodium picrate and picric acid has also been determined. The data are given in Tables XIV and XV.

In Tables IX and X, the constants for FREUNDLICH's adsorption isotherm show that the two components of lysine picrate as well as arginine picrate are adsorbed by charcoal in equimolecular ratio within the limits of experimental error. Arginine

TABLE IX

*Lysine monopicrate.*

| mol. conc. of solution | equilibrium conc. $C$ | $\log C$ | Amount adsorbed by 1 g of charcoal: experimental $x/m$ | experimental $\log x/m$ | calculated $x/m$ |
|---|---|---|---|---|---|
| *Lysine cation:* * $\log K = 0.627$, * $1/n = 0.2208$ | | | | | |
| 0.01750 | 0.01545 | −1.8069 | 1.680 | 0.2254 | 1.687 |
| 0.008736 | 0.007059 | −2.1512 | 1.423 | 0.1531 | 1.420 |
| 0.006988 | 0.005353 | −2 2714 | 1.336 | 0.1258 | 1.335 |
| 0.004368 | 0.002935 | −2.5324 | 1.174 | 0.0697 | 1.169 |
| 0.002184 | 0.001050 | −2.9797 | 0.926 | −0.0333 | 0.9292 |
| *Picrate anion:* $\log K = 0.673$, $1/n = 0.2333$ | | | | | |
| 0.01750 | 0.01534 | −1.8142 | 1.771 | 0.2481 | 1.777 |
| 0.008736 | 0.006958 | −2.1575 | 1.457 | 0.1635 | 1.479 |
| 0.006988 | 0.005231 | −2.2814 | 1.358 | 0.1329 | 1.386 |
| 0.004368 | 0.002860 | −2.5446 | 1.235 | 0.0919 | 1.201 |
| 0.002184 | 0.001021 | −2.9910 | 0.953 | −0.0208 | 0.924 |

TABLE X

*Arginine monopicrate.*

| mol. conc. of solution | equilibrium conc. $C$ | $\log C$ | Amount adsorbed by 1 g of charcoal: experimental $x/m$ | experimental $\log x/m$ | calculated $x/m$ |
|---|---|---|---|---|---|
| *Arginine cation:* $\log K = 0.661$, $1/n = 0.2040$ | | | | | |
| 0.009670 | 0.007570 | −2.1209 | 1.720 | 0.2355 | 1.692 |
| 0.004841 | 0.003050 | −2.5157 | 1.470 | 0.1673 | 1.405 |
| 0.003871 | 0.002268 | −2.6444 | 1.314 | 0.1186 | 1.323 |
| 0.002420 | 0.001030 | −2.9872 | 1.139 | 0.0565 | 1.124 |
| 0.001933 | 0.0006676 | −3.1755 | 1.041 | 0.0174 | 1.031 |
| *Picrate anion:* $\log K = 0.657$, $1/n = 0.2020$ | | | | | |
| 0.009670 | 0.007603 | −2.1190 | 1.694 | 0.2290 | 1.699 |
| 0.004841 | 0.003077 | −2.5119 | 1.446 | 0.1602 | 1.412 |
| 0.003871 | 0.002288 | −2.6416 | 1.297 | 0.1130 | 1.329 |
| 0.002420 | 0.001038 | −2.9839 | 1.133 | 0.0542 | 1.134 |
| 0.001933 | 0.0006801 | −3.1674 | 1.026 | 0.0111 | 1.041 |

* $\log K$ and $1/n$ are the constants for FREUNDLICH's adsorption isotherm, viz., $\log x/m = \log K + 1/n \log C$, where $x/m$ is the weight (millimol) of substance adsorbed per gram of charcoal, $C$ is the equilibrium concentration, and $K$ and $1/n$ are empirical constants.

TABLE XI

*Histidine dipicrate.*

| mol. conc. of solution | equilibrium conc. $C$ | $\log C$ | Amount adsorbed by 1 g of charcoal: experimental $x/m$ | experimental $\log x/m$ | calculated $x/m$ |
|---|---|---|---|---|---|
| *Histidine cation:* $\log K = 0.146$, $1/n = 0.0787$ | | | | | |
| 0.009600 | 0.008440 | −2.0737 | 0.973 | −0.0119 | 0.962 |
| 0.007680 | 0.006550 | −2.1838 | 0.926 | −0.0333 | 0.934 |
| 0.004800 | 0.003700 | −2.4318 | 0.902 | −0.0448 | 0.901 |
| 0.003600 | 0.002550 | −2.5935 | 0.861 | −0.0650 | 0.875 |
| 0.001755 | 0.007815 | −3.1070 | 0.798 | −0.0980 | 0.799 |
| *Picrate anion:* $\log K = 0.638$, $1/n = 0.1289$ | | | | | |
| 0.01920 | 0.01608 | −1.7938 | 2.557 | 0.4078 | 2.551 |
| 0.01536 | 0.01230 | −1.9101 | 2 508 | 0.3993 | 2.465 |
| 0.00960 | 0.006818 | −2.1664 | 2.280 | 0.3579 | 2.281 |
| 0.00720 | 0.004614 | −2.3359 | 2.120 | 0.3263 | 2.189 |
| 0.003510 | 0.001263 | −2.8986 | 1.844 | 0.2657 | 1.837 |

TABLE XII

*Canavanine dipicrate.*

| mol. conc. of solution | equilibrium conc. $C$ | $\log C$ | Amount adsorbed by 1 g of charcoal: experimental $x/m$ | experimental $\log x/m$ | calculated $x/m$ |
|---|---|---|---|---|---|
| *Canavanine cation:* $\log K = 0.295$, $1/n = 0.1226$ | | | | | |
| 0 005760 | 0.004500 | −2.3468 | 1.033 | −0.0140 | 1.018 |
| 0.004800 | 0.003620 | −2.4413 | 0.967 | −0.0146 | 0.990 |
| 0.003589 | 0.002438 | −2.6130 | 0.943 | −0.0255 | 0.943 |
| 0.002400 | 0.001323 | −2.8784 | 0.883 | −0.0540 | 0.875 |
| 0.001920 | 0.0009177 | −3.0370 | 0.821 | −0.0857 | 0.837 |
| *Picrate anion:* $\log K = 0.538$, $1/n = 0.1281$ | | | | | |
| 0.01152 | 0.009320 | −2.0306 | 1.803 | 0.2560 | |
| 0.009600 | 0.007350 | −2.1337 | 1.844 | 0.2657 | 1.813 |
| 0.007178 | 0.005048 | −2.2969 | 1.746 | 0.2420 | 1.753 |
| 0.004800 | 0.002824 | −2.5492 | 1.620 | 0.2094 | 1.628 |
| 0.003840 | 0.001943 | −2.7115 | 1.558 | 0.1925 | 1.551 |

TABLE XIII

*Canaline dipicrate.*

| mol. conc. of solution | equilibrium conc. $C$ | $\log C$ | Amount adsorbed by 1 g of charcoal: experimental $x/m$ | experimental $\log x/m$ | calculated $x/m$ |
|---|---|---|---|---|---|
| *Canaline cation:* $\log K = 1.168$, $^1/_n = 0.4626$ | | | | | |
| 0.007680 | 0.005953 | −2.2253 | 1.415 | 0.1507 | 1.377 |
| 0.004800 | 0.003575 | −2.4457 | 1.050 | 0.0212 | 1.089 |
| 0.002400 | 0.001489 | −2.8270 | 0.7767 | −0.1269 | 0.7240 |
| 0.001920 | 0.001142 | −2.9423 | 0.6378 | −0.1954 | 0.6410 |
| *Picrate anion:* $\log K = 0.610$, $^1/_n = 0.1301$ | | | | | |
| 0.01536 | 0.01252 | −1.9024 | 2.327 | 0.3669 | 2.304 |
| 0.00960 | 0.006965 | −2.1571 | 2.160 | 0.3344 | 2.135 |
| 0.00480 | 0.002504 | −2.6041 | 1.882 | 0.2746 | 1.868 |
| 0.00384 | 0.001686 | −2.7705 | 1.757 | 0.2448 | 1.776 |

picrate is adsorbed slightly better than lysine picrate. The difference is not so marked as that which was found by T. ITO* between the hydrochloride of arginine and of lysine. This may be accounted for by the following assumption; the combination of charcoal with the picrate of arginine or lysine may take place at the picrate anion, presumably at the nitro group, whereas the combination of charcoal with the corresponding hydrochloride may take place at the cation of amino acid. This assumption will be fully discussed later on.

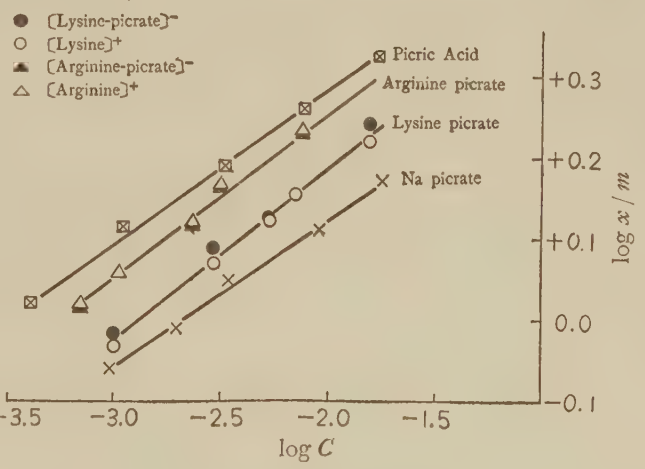



FIG. 7. Adsorption isotherms for picrate anions and amino acid cations.

* T. ITO[30] found that arginine hydrochloride is adsorbed five times as much as lysine hydrochloride.

Tables XI, XII, and XIII indicate that the dipicrates of histidine and canaline are adsorbed in a different manner from the monopicrate. From the constants for the adsorption isotherm, it will be seen that, among these dipicrates, only canavanine dipicrate is adsorbed in the molecular proportion of two components, or in other words, adsorbed as the dipicrate molecule that it is. In histidine dipicrate, on the other hand, the histidine cation is adsorbed at a ratio less than half of the picrate anion. This can also be explained by the foregoing assumption that the center of adsorption of the picrate consists in the picrate anion. The following diagrams will illustrate this assumption.

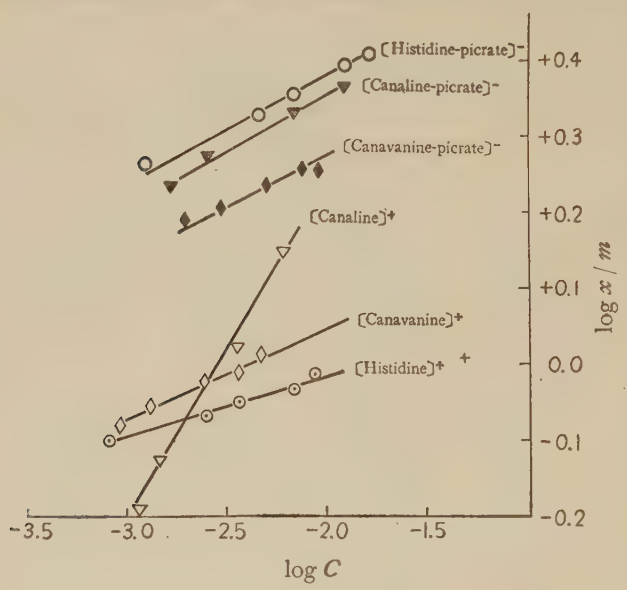
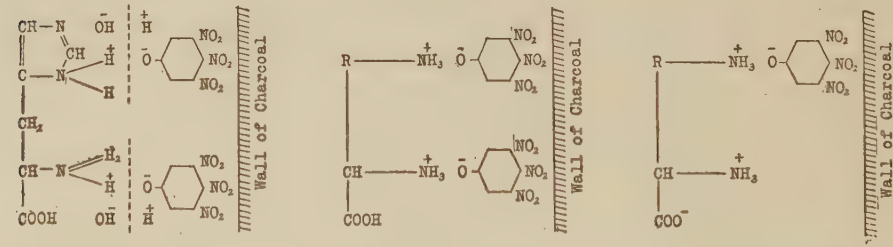



FIG. 8. Adsorption isotherms for picrate anions and amino acid cations.




(I) Histidine dipicrate (II) Canavanine dipicrate (III) Arginine- or Lysine-monopicrate

Due to the small basic dissociation constants of histidine and the large dissocation constant of picric acid, histidine dipicrate in aqueous solution hydrolyzes to a certain extent. The hydrolyzed part of histidine dipicrate may be shown as in diagram I. It will be seen that two components of the hydrolyzed part may exist indepen-

dently, almost without affinity between them. The picric acid molecule which is thus formed will enter the adsorption layer of charcoal, while the free amino acid formed at the same time loses the affinity with picric acid molecule, remaining outside the adsorption layer. It may be said, accordingly, that whether or not the adsorption takes place in molecular proportion of two components depends upon the dimension* of the dissociation constant of the diamino acid under question. This is why basic amino acids with stronger basicity than histidine are adsorbed in the molecular proportion of two components. The adsorption of canavanine dipicrate can be illustrated by diagram II, and that of the monopicrate of lysine or arginine by diagram III.

Canaline picrate shows an abnormal behavior in adsorption. The adsorption of this picrate anion takes place in a similar way as that of histidine dipicrate or canavanine dipicrate. In the concentrations lower than 0.0048 mol., the adsorption of canaline cation is similar to histidine cation, but it increases very much with the increase of concentration of canaline dipicrate, namely the intensity factor of the adsorption isotherm for canaline cation is more than twice as much as that for amino acid cations of other picrates. The reason for this abnormality is not clear.

It is to be emphasized here that there is a strong evidence to support the foregoing assumption that the adsorption of picrate of diamino acid takes place at its picrate anion. The aqueous solutions of monohydrochloride of histidine, lysine, and arginine were treated with charcoal, but only a trace of them was adsorbed. When, however, a charcoal which had adsorbed picric acid was shaken with the solution of the monohydrochloride, a considerable adsorption of diamino acid took place. This will be clearly seen from the following example.

| Kinds of charcoal | Histidine-HCl (0.0106 mol) | Lysine-HCl (0.0152 mol) | Arginine-HCl (0.0150 mol) |
|---|---|---|---|
| (a) | 0.11 | 0.07 | 0.18 |
| (b) | 0.31 | 1.70 | 1.85 |

(a): charcoal without any treatment.

(b): charcoal shaken with the solution of picric acid, washed twice with distilled water, and then dried.

This experiment clearly indicates that the diamino acid or its monovalent cation has

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* $pK'_{b_1}$ and $pK'_{b_2}$ values for these diamino acids are as follows:
4.96 and 11.98 for arginine,[5] 8.00 and 12.18 for histidine,[6]
5.06 and 11.82 for lysine,[6] 7.40 and 11.45 for canavanine,[35]
10.30 and 11.55 for canaline.[35]

a very small affinity with charcoal, and that, if the surface of charcoal is charged with picric acid, diamino acid can enter, by the aid of affinity with picric acid, the adsorption layer of charcoal in an amount equivalent to picric acid which has already been adsorbed (except histidine). Making use of ITO's data on the adsorption of diamino acid hydrochlorides by charcoal, an approximate comparison can be made between adsorptions of diamino acid in the form of picrate and in the form of hydrochloride.

| | The amount adsorbed (millimol / g charcoal) | | |
|---|---|---|---|
| | Histidine | Lysine | Arginine |
| Hydrochloride | 0.31 | 0.08 | 0.39 |
| Picrate | 1.11 | 2.20 | 2.51 |
| $\frac{\text{Picrate}}{\text{Hydrochloride}} =$ | 3.6 | 27 | 6.4 |

TABLE XIV

*Sodium picrate.*

*Picrate anion :* $\log K = 0.470$, $^1/_n = 0.1777$

| mol. conc. of solution | equilibrium conc. $C$ | $\log C$ | Amount adsorbed by 1 g of charcoal | | |
|---|---|---|---|---|---|
| | | | experimental | | calculated |
| | | | $x / m$ | $\log x / m$ | $x / m$ |
| 0.01923 | 0.01774 | −1.7510 | 1.486 | 0.1713 | 1.475 |
| 0.01047 | 0.009163 | −2.0380 | 1.304 | 0.1152 | 1.328 |
| 0.004583 | 0.003458 | −2.4612 | 1.125 | 0.0510 | 1.120 |
| 0.002856 | 0.001884 | −2.7238 | 0.9720 | −0.0123 | 1.009 |
| 0.001829 | 0.0009550 | −3.0120 | 0.8745 | −0.0583 | 0.8974 |

TABLE XV

*Picric acid.*

*Picric acid anion :* $\log K = 0.648$, $^1/_n = 0.1840$

| mol. conc. of solution | equilibrium conc. $C$ | $\log C$ | Amount adsorbed by 1 g of charcoal | | |
|---|---|---|---|---|---|
| | | | experimental | | calculated |
| | | | $x / m$ | $\log x / m$ | $x / m$ |
| 0.01920 | 0.01710 | −1.7645 | 2.100 | 0.3222 | 2.109 |
| 0.00960 | 0.007780 | −2.1090 | 1.820 | 0.2601 | 1.820 |
| 0.00480 | 0.003248 | −2.4884 | 1.552 | 0.1909 | 1.549 |
| 0.00240 | 0.001094 | −2.9609 | 1.306 | 0.1148 | 1.272 |
| 0.00145 | 0.0004001 | −3.3971 | 1.050 | 0 0212 | 1.054 |

From the data given in Tables XIV and XV, it is understood that the increase in acidity will increase the adsorption of the picric anion. T. Ito[30] pointed out the importance of the hydrogen ion concentration upon the adsorption of diamino acids. Since it has been determined that, in the adsorption of diamino acid picrate, the picrate anion plays an important rôle, the effect of pH upon the adsorption of picric acid anion on charcoal has been investigated. The result is shown in Table XVI.

Table XVI

*The Effect of pH on Degree of Adsorption of Picric Acid.*

| pH | Molar conc. of solution | Equilibrium conc. $C$ | Amount adsorbed by 1 g charcoal | |
|---|---|---|---|---|
| | | | $x$ | $x/m$ |
| 8.03 | 0.004737 | 0.003482 | 0.001255 | 1.126 |
| 7.15 | 〃 | 0.003475 | 0.001262 | 1.132 |
| 4.50 | 〃 | 0.003458 | 0.001379 | 1.147 |
| 3.23 | 〃 | 0.003342 | 0.001396 | 1.252 |
| 2 80 | 〃 | 0.003200 | 0.001537 | 1.379 |
| 2.60 | 〃 | 0.003154 | 0.001583 | 1.420 |
| 2.34 | 〃 | 0.002864 | 0.001873 | 1.680 |
| 2.05 | 〃 | 0.002830 | 0.001907 | 1.711 |
| 1.00 | 〃 | 0.002800 | 0.001937 | 1.721 |

As the pK′ value of picric acid is 1.2,[36] the sudden increase of adsorption with the decrease of pH value must be ascribed to the formation of picric acid. It remains uncertain whether this increase is due to the undissociated molecule of picric acid, or picric acid molecule as a whole. The pH range of aqueous solutions of various concentrations of diamino acid picrates under examination was found as following: lysine picrate, $5.5 \sim 6.5$; arginine picrate, $3.5 \sim 5$; histidine picrate, canavanine picrate, canaline picrate, and picric acid, $2 \sim 3$. It can be explained from these data that the picrate anion of histidine-, canavanine-, and canaline-dipicrate is more largely adsorbed than that of lysine- and arginine-monopicrate. But when the data for the adsorption of picrate anion of histidine or canaline is compared with that for the adsorption of picric acid, these adsorptions having taken place within almost the same range of pH, it will be seen that the effect of the diamino acid cation upon the adsorption of the corresponding picrate anion can not be neglected.

From the data given above, the following order of picrates can be made as to the degree of adsorption of the picrate anions by charcoal.

Histidine dipicrate > Canaline dipicrate > Canavanine dipicrate > picric acid > Arginine monopicrate > Lysine monopicrate > Sodium picrate.

Regarding the degree of the adsorption of diamino acid cations, the following order can also be made.

Arginine monopicrate > Lysine monopicrate > Canavanine dipicrate > Histidine dipicrate.

The data showing that charcoal can adsorb diamino acids in the form of picrates much better than in the form of hydrochlorides, gives rise to a matter of particular interest; namely, the applying of this phenomenon to the isolation of diamino acids. Further studies of the effect of pH upon the adsorption of salt of diamino acids with certain organic coloring compounds will bring promising results.

## Summary

1. The adsorption by charcoal of each component of several diamino acid-picrates conforms to FREUNDLICH'S adsorption isotherm. The constants of the equation for each picrate have been calculated.

2. The monopicrate of arginine or lysine is adsorbed in molecular proportion of its components. In the case of histidine dipicrate, the histidine cation is adsorbed in an amount less than half of the picrate anion adsorbed. The adsorption of canaline cation shows a remarkable increase with the increase of the concentration of canaline dipicrate.

3. Utilizing ITO'S data, it is calculated approximately that, in the form of picrate, histidine is adsorbed 3.6 times, arginine 6.4 times, lysine 27 times as much as respective diamino acid in the form of hydrochloride.

4. The probable mechanism of adsorption of these picrates by charcoal has been discussed. The facts which have been obtained in the present investigation can be explained by assuming that the combination of diamino acid picrates with charcoal does not take place at the amino acid cation but at the picrate anion.

5. The factors governing the adsorption of the diamino acid-picrate are the concentration of hydrogen ion of the solution and the kinds of diamino acid cation.

6. The degree of adsorption of picrate of diamino acid under examination can be shown in the following order:

For the adsorption of picrate anion,

Histidine dipicrate > Canaline dipicrate > Canavanine dipicrate > Picric acid > Arginine monopicrate > Lysine monopicrate > Sodium picrate.

For the adsorption of diamino acid cation,

Arginine monopicrate > Lysine monopicrate > Canavanine dipicrate > Histidine dipicrate.

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# PART II

# STUDIES ON THE METHODS FOR ISOLATION AND DETERMINATION OF CERTAIN AMINO ACIDS

## CHAPTER V

## A New Method for the Estimation of Tryptophane

A number of methods have been presented for the determination of tryptophane; the gravimetric method according to HOPKINS and COLE[37] as well as VON MORACZEWSKI,[38] and the colorimetric methods, in which use has been made of bromine adsorption test,[39] ADAMKIEWICZ reaction,[40] nitrosoindol reaction,[41] and the reaction with phenol reagent[42] as well as EHRLICH's reagent.[43] The coloration formed by the reaction of tryptophane with the EHRLICH reagent which was first found by ROHDE,[43] has advantages of being specific and stable. The method which takes advantage of this specific color reaction has been examined by such investigators as HERZFELD,[44] THOMAS,[45] and MAY and ROSE,[46] the last authors' method having been extensively used.

In their method the protoin is dissolved in 19 % hydrochloric acid which contains the EHRLICH reagent, incubated at 35° for 24 hours, and then allowed to stand for an additional 40 hours st room temperature. The blue color which is thus produced is matched against a similar color obtained by treating casein in an identical manner. In this procedure such factors as the particular preparation of casein used for the standard and the room temperature may be uncontrolled variables. MAY and ROSE assumed the tryptophane content of casein to be 1.5 %. It is based on the data of HOPKINS and COLE. Other values have been reported: 0.51 % (HERZFELD),[44] 1.7-1.8 % (THOMAS),[45] 2.02 % (FÜRTH and NOBEL),[47] 1.4 % (FOLIN and CIOCALTEAU),[42] and 2.24 % (HOLM and GREENBANK),[48] respectively. The uncertainty as to the tryptophane content of casein makes this protein of questionable value as a standard.

It was found in the present experiment that, by using pure tryptophane as a standard, divergent tryptophane values for casein were obtained depending on whether the determinations were carried out in summer or in winter, namely 1.7 per cent in the former and 3.1 per cent in the latter. It was further observed that the development of the color does not proceed at the same rate when casein and tryptophane are treated with the reagent. Tryptophane reacts more slowly.

As to the effect of temperature on the reaction between tryptophane and the EHRLICH reagent, HOLM and GREENBANK[48] have investigated it by using the color of copper ammonia complex as a comparison standard. In their experiment, the maximum color intensity was obtained in 8 days at 45°, in 12 days at 37°, and in 20 days at 25°. The present data are not entirely in accord with those reported by HOLM and GREENBANK. In the present experiment the maximum color was obtained in about 80 hours at 37°, and 120 hours at 30°. At 45° both casein and tryptophane react more rapidly than at either 37° or 30°; however, at 45° both substances show only 60 ~ 80 % of the maximum color intensity of that which was obtained at either of the two lower temperatures. Apparently considerable destruction of tryptophane takes place at 45°. At 30° the color is fairly stable and continues so for a period of 48 hours after reaching the maximum intensity. Accordingly, the accurate determination of tryptophane will only be carried out by permitting the color development at the optimum condition and by adopting a proper standard instead of casein.

In the present work the attempt was made to use some color standard whose color is comparable to that given by the color reaction and is furthermore easy to obtain and reproduce at any laboratory. HERZFELD[44] has shown that the color of the copper ammonium complex salt is similar to that obtained when tryptophane is treated with the EHRLICH reagent. An objection to the use of the former color standard are the fumes of ammonium chloride which are formed when the protein or tryptophane which is treated with the hydrochloric acid-containing reagent is brought near the ammonia-containing copper reagent. In the present experiments it was found that the color produced by reduced phosphomolybdate which is made use of in the determination of phosphorus was quite similar to the color of the condensation product formed when tryptophane is treated with the EHRLICH reagent. For the estimation of tryptophane the blue color given by a standard phosphate solution prepared essentially according to LOHMANN and JENDRASSIK'S[49] modification of FISKE and SUBBAROW'S method was adopted as a color standard.

## Method

*Preparation of Reagent and Standard.*

(1) Standard phosphate solution: 0.4394 g of $KH_2PO_4$ is dissolved in a liter of water, i. e., 1 cc is equivalent to 0.1 mg Phosphorus.

(2) Molybdate solution: 2.5 % solution of ammonium molybdate in 5 *N* sulfuric acid.

(3) Eikonogen solution: 0.5 g of Eikonogen (amino naphthol sulfonic acid is dissolved in a mixture of 195 cc of 15 % $NaHSO_3$ solution and 5 cc of 20 % $Na_2SO_3$ solution.

(4) EHRLICH reagent: 5 % solution of *p*-dimethylaminobenzaldehyde in 10 % sulfuric acid.

(5) 19 % HCl

(6) 0.2 % NaOH

The standard color is prepared by pipetting 10 cc of the standard phosphate solution, 5 cc of the ammonium molybdate solution, and 1 cc of the Eikonogen solution into a 25 cc volumetric flask, the mixture is warmed in a water bath at 37° for 15 minutes, then cooled to room temperature with tap water, and brought to volume by the addition of distilled water. This solution is diluted ten times and used as the color standard. It can be accurately reproduced and does not change appreciably over a 24-hour period. The color of the phosphate mixture is just equivalent to the color of a mixture which contained 0.860 mg of tryptophane dissolved in 10 cc of distilled water to which were added 2 cc of 0.2 % Na OH solution, 90 cc of 19 % HCl, and 1 cc of EHRLICH reagent, and incubated at 30° for 6 ~ 7 days, i. e., the period which gave the maximum color intensity.

*The Method for the Determination.*

About 50 mg of sample protein which is pulverized into a fine powder are brought into solution by warming at about 50° with 2 cc of 0.2 % Na OH[50]. After cooling, 100 cc of 19 % HCl and 1 cc of the EHRLICH reagent are added and the solution is incubated at 30° until it gives the maximum color intensity. It takes ordinarily 6 ~ 7 days for the development of the maximum color intensity; however, further incubation and daily comparisons with the standard color are necessary to ascertain the development of the maximum color intensity. The content of tryptophane in the protein may be calculated by the following formula:

$$0.00086 \times \frac{a}{b} \times \frac{100}{S} = T$$

where, $a$: the reading of the colorimeter tube containing the standard solution,
$b$: the reading of the colorimeter tube containing the sample solution,
$S$: the weight of moisture-free sample protein,
$T$: the content of tryptophane %.

## Experimental

### I. Standardization of the Tryptophane Content of Casein by Pure Tryptophane Using the Orginal May and Rose's Method

As a tryptophane standard, Schuchardt's (m. p. 275°) and Grübler's (m. p. 268°) products were used. Both Merck's Hammarsten casein and the tryptophane were treated with the Ehrlich reagent according to May and Rose's method. As is shown in Table XVII, the results are quite variable and depend upon the room

Table XVII

*The Tryptophane Content of Casein at Different Temperatures.*

| Room temp. (°C) | Weight of sample casein (g) | Reading of colorimeter | | Tryptophane content (%) |
|---|---|---|---|---|
| | | Tryptophane | Casein | |
| 25 ± 2 | 0.0488 | *20 | 18.8 | 1.86 |
| 25 ± 2 | 0.0687 | †20 | 18.0 | 1.61 |
| 15 ± 3 | 0.0528 | ††20 | 9.8 | 3.10 |

* 0.851 mg Schuchardt's product. † 0.998 mg Grübler's. †† 0.803 mg Schuchardt's.

temperature at which the experiments are carried out. It is further noticed that the development of the color does not proceed at the same rate when casein and tryptophane are treated with the reagent.

### II. Effect of Temperature upon the Development of Color Given by Casein and Tryptophane When Treated with Ehrlich's Reagent

In an attempt to examine the course of color development and a maximum color intensity, was used the color of reduced phosphomolybdate as a comparison standard. The mode of preparation of the standard is given in the foregoing description of the method. The course of development of the tryptophane color in casein (Hammarsten) and tryptophane (Schuchardt) was followed until the maximum point was obtained. The results are shown in Fig. 9. In plotting the

results, the minimum colorimeter reading which was observed at 30° was adopted as 100 per cent color intensity.

When incubated at 45°, the development of the color given by pure tryptophane was rapid and the maximum color intensity was obtained after a 24-hour period of incubation. This, however, corresponded to about 60 per cent of the maximum color intensity at 37° or 30°. The coloration formed at 45° gradually

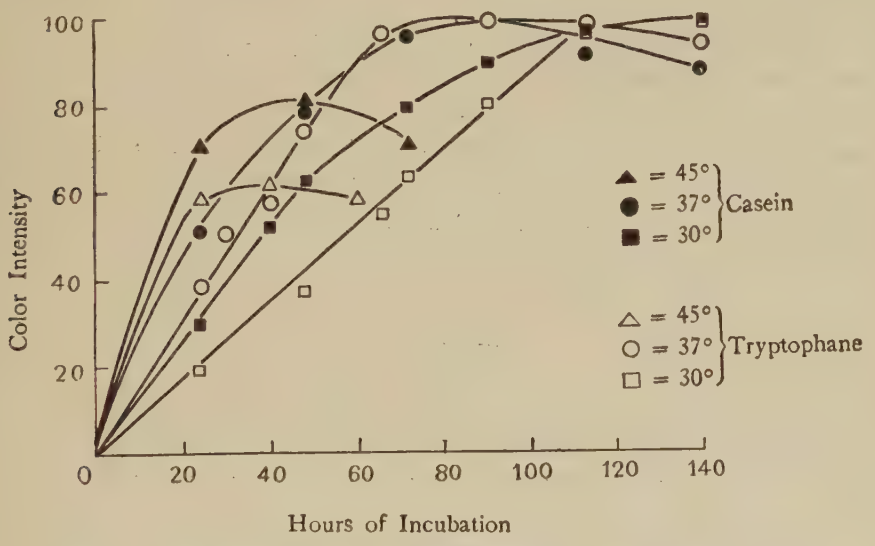


Fig. 9. The Course of the Color Development at Various Temperatures

turned into violet after reaching the maximum intensity. A similar phenomenon was also observed in casein. At 30°, on the other hand, the maximum color intensity was stable over at least 48 hours after reaching the maximum intensity. Accordingly, 30° was chosen for permitting the color reaction to take place.

### III. Standardization of the Color of Reduced Phosphomolybdate by Pure Tryptophane

10 cc of an aqueous solution containing an appropriate amount of tryptophane (Schuchardt) were mixed with 2 cc of 0.2% NaOH, 90 cc of 19% HCl, and 1 cc of the Ehrlich reagent. This mixture was incubated at 30° and daily comparisons of the color developed were made with the color of reduced phosphomolybdate at several time-periods. The results are given in Table XVIII.

TABLE XVIII

*The Course of Development of the Tryptophane Color When the Inorganic Color Standard Was Set at 20 on the Colorimeter.*

| Sample \ Days of incubation | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| 0.70 mg tryptophane | | 60.6 | 39.2 | 31.0 | 25.0 | 24.9 | | | |
| 0.83 mg tryptophane | 78.2 | 36.5 | 25.9 | 24.4 | 23.3 | — | 20.7 | 21.8 | 22.3 |
| | 67.4 | — | 24.9 | 24.4 | 23.6 | — | 20.9 | 21.9 | 23.0 |

Table XVIII indicates that the maximum color intensities obtained in two separate experiments are in good agreement, although the course of the color development differs to some extents. The data also show that the color of the reduced phosphomolybdate is just equivalent to the color given by a solution of tryptophane containing 0.860 mg per 103 cc when treated with the EHRLICH reagent.

TABLE XIX

*The Course of Development of the Tryptophane Color in Various Kinds of Proteins When the Inorganic Color Standard Was Set at 20 on the Colorimeter.*

| Protein | Sample taken (mg) | Days of Incubation | | | | | | | Tryptophane Content % | Folin and Ciocalteau Values |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 3 | 4 | 5 | 6 | 8 | 10 | 12 | | |
| Casein | 50.2 | 29.7 | 23.8 | 21.6 | 21.4 | 21.5 | 21.6 | 25.0 | 1.60 | 1.40 |
| Edestin | 51.0 | 28.6 | 25.6 | 23.2 | 23.4 | 22.2 | 22.4 | 23.9 | 1.52 | 1.51 |
| Egg Albumin | 64.2 | 31.8 | 28.4 | 23.7 | 23.3 | 21.8 | 21.0 | 20.9 | 1.23 | 1.23 |
| *Protein of Soy Bean | 50.0 | 54.6 | 36.6 | 31.5 | 32.7 | 30.7 | 30.4 | 33.6 | 1.13 | |
| " " Silk-worm Pupa | 55.9 | 56.8 | 33.1 | 26.8 | 25.6 | 23.6 | 22.1 | 22.6 | 1.40 | |
| *Muscle Protein of Bonito | 50.0 | 63.8 | 46.4 | 38.8 | 37.4 | 33.7 | 31.0 | 30.0 | 1.15 | |
| Whale | 50.2 | 54.2 | 38.4 | 33.3 | 33.5 | 31.3 | 28.9 | 28.1 | 1.22 | |
| Sardine | 50.0 | 62.8 | 41.0 | 34.5 | 33.8 | 31.1 | 29.7 | 29.4 | 1.17 | |
| Chicken | 50.0 | 50.6 | 44.6 | 37.2 | 35.4 | 33.3 | 31.1 | 30.6 | 1.12 | |

* The preparation of these mixed proteins is described in Chapter X, pp. 193.

### IV. Determination of Tryptophane in Various Kinds of Protein by the Present Method

With the object of applying the present method to the determination of tryptophane in proteins, the time period for the arrival of the maximum color intensity was examined, using several kinds of protein. The results are given in Table XIX.

Tryptophane content of Merck's Hammarsten casein was found to be 1.6 %. This is somewhat higher than the figure reported by Folin and Ciocalteau.[42] Of the other proteins given in Table XIX, the percentage of tryptophane in edestin and egg albumin agrees with the values obtained by the latter workers. The data given in Table XIX show that such mixed proteins as those of muscle should be incubated for a longer period than the more simple proteins in order to obtain the maximum intensity of the color. This is probably due to the difficulty of effecting complete solution of the protein material which contains muscle fibre. In this connection Thomas[45] has reported that, when the sample was carefully ground in an agate mortar before subjecting it to digestion by trypsin, the tryptophane value of casein rose 1.3 to 1.6 % when the Fasal[40] method was employed. It is obvious, in order to obtain accurate results, that complete solution of the protein in question is a necessary prerequisite. In the case of finely ground proteins which are difficultly soluble, about 8 days of incubation are necessary in order to reach the maximum color intensity. It is advisable that daily comparisons of the sample solution be made after the sixth day in order to ascertain the point of maximum color intensity.

### Summary

1. The sources of the error in May and Rose's method have been examined and a modification for it has been devised.

2. The incubation of the reaction mixture at 30° has been found to be optimum for the development as well as the stability of the color formed by the reaction between tryptophane and the Ehrlich reagent.

3. It has been indicated that the uncertainty as to the tryptophane content of casein makes this protein of questionable value as a standard. Hence, the blue color given by a standard phosphate solution prepared essentially according to Lohmann and Jendrassik's modification of Fiske and Subbarow's method has been adopted as a color standard.

4. About 6~7 days of incubation are necessary in order to reach the maximum color intensity.

## CHAPTER VI

## The Distribution of Methionine in Several Proteins

Methionine, a sulphur containing amino acid which was discovered by MUELLER,[51] has been found to be present in casein, egg albumin, edestin, wool (?), gelatine (?), fibrin, protein of rice bran, and brewery yeast, by several authors, such as MUELLER,[52] ODAKÉ,[53] DU VIGNEAUD and MEYER,[54] and PIRIE.[55] BÄRNSTEIN[56] determined the methylthiol group, characteristic of methionine, in several proteins. Calculating from the results he assumed that methionine is widely distributed in many kinds of protein. Recently, JACKSON and BLOCK,[57] and T. E. WEICHSELBAUM, M. B. WEICHSELBAUM and STEWART[58] reported that the methionine, like cystine, is capable of stimulating growth in albino rats subsisting on a basal diet poor in cystine.

As far as we know, this amino acid has not yet been isolated in a pure state from the proteins of sardine- and whale-muscle, silk-worm pupa, and soy bean. The present work deals with the isolation of methionine in crystalline form from these proteins and casein, and with the identification of it by elementary analysis both of the free amino acid and of its derivatives, i. e., copper salt and $\alpha$-naphthyl-isocyanate.

### I. PREPARATION OF SAMPLE PROTEINS AND ISOLATION OF METHIONINE

The sample proteins, from which methionine was isolated, were prepared according to the same method as is described in Chapter X, pp. 198. The sample casein is a commercial preparation (Katayama and Co.).

In order to isolate methionine from these proteins, MUELLER'S[52] method slightly modified by ODAKÉ was adopted. 200 g of the sample protein are mixed with a solution of 400 g concentrated sulfuric acid and 1300 cc water. The mixture is boiled until it shows no biuret reaction. To the hydrolyzate, after being neutralized by caustic soda, is added a sufficient amount of mercuric sulfate solution which consists of 144 cc concentrated sulfuric acid, 1110 cc water, and 200 g mercuric sulfate. Then, the acidity of the mixture is reduced to slightly acidic by adding caustic soda. After standing in ice box overnight, the precipitate formed is separated and washed several times by grinding it in a mortar with warm distilled water. The washed precipitate is mixed with a solution of baryta, care

being taken to make the concentration of the latter two per cent. The residue is further extracted several times with 2% baryta at 50°. From the extract is removed mercuric ion by hydrogen sulfide, and sulfate ion by baryta. The filtrate thus obtained is concentrated in vacuo and then treated with a hot saturated solution of mercuric chloride. The gum-like precipitate is ground with quartz sand, suspended in water, and treated with hydrogen sulfide to remove mercuric ion. The filtrate is freed from hydrochloric acid by concentrating in vacuo and adding silver oxide, excess of which is removed as sulfide. The crude methionine fraction is finally obtained from the last filtrate by concentrating to a small volume and adding warm absolute alcohol. The crude methionine crystal can be purified by repeating the procedure of precipitating with mercuric chloride, decomposing by hydrogen sulfide, and then crystallizing from alcohol.

The analytical results of the sample protein and the yield of crystalline methionine therefrom are recorded in Table XX.

TABLE XX *Analysis of Sample Protein and Yield of Methinine.*

| Sample protein | Moisture % | Ash % | Nitrogen % | Sample protein taken (g) | Methionine found (g) | Yield of Methionine % |
|---|---|---|---|---|---|---|
| Muscle protein of sardine | 12.14 | 1.04 | 13.67 | 200 | 0.897 | 0.45 |
| Muscle protein of whale | 12.17 | 0.35 | 14.04 | 400 | 1.314 | 0.32 |
| Protein of silk-worm pupa | 8.19 | 0.93 | 12.85 | 200<br>200 | 0.788<br>0.615 | 0.39<br>0.31 |
| Protein of soy bean | 10.96 | 2.78 | 12.50 | 400 | 0.286 | 0.07 |
| Casein | 9.80 | 1.42 | 13.38 | 200 | 0.720 | 0.36 |

## II. PROPERTIES OF ISOLATED METHIONINE

Heating in open capillary tube, the isolated crystals began to colour at 230 ~ 240°C; melted with simultaneous decomposition at the following temperatures.

| | |
|---|---|
| Crystal from sardine muscle protein | 267 ~ 269°C |
| Crystal from whale muscle protein | 267 ~ 269°C |
| Crystal from the protein of silk-worm pupa | 265 ~ 268°C |
| Crystal from the protein of soy bean | 268 ~ 269°C |
| Crystal from casein | 268 ~ 270°C |

All these crystals are very soluble in water, while insoluble in alcohol or ether; they form sparingly soluble copper salt as well as insoluble mercuric salt, but can be precipitated neither by phosphotungstic acid nor picric acid. Ninhydrin reaction is strongly positive, while color reaction such as biuret, millon, and nitroprussid are all negative.

## III. Identification of the Isolated Crystals

The free amino acid crystal, isolated from the five proteins aforementioned, and its derivatives were identified by the elementary analysis.

A. *Free methionine* $C_5H_{11}NSO_2$

The crystals dried at 100°C in vacuo were subjected to the micro analysis for carbon, hydrogen, and nitrogen with the results given in Tables XXI and XXII.

Table XXI *Analysis for Carbon and Hydrogen of the free amino acid.*

| C and H / Sample protein | Sample taken (mg) | $CO_2$ (mg) | $H_2O$ (mg) | C % | H % |
|---|---|---|---|---|---|
| Muscle protein of sardine | 3.708<br>3.930 | 5.400<br>5.740 | 2.530<br>2.686 | 39.71<br>39.82 | 7.63<br>7.65 |
| Muscle protein of whale | 4.852<br>4.445 | 7.231<br>6.665 | 3.315<br>3.125 | 40.64<br>40.89 | 7.65<br>7.87 |
| Protein of silk-worm pupa | 3.485<br>4.067 | 5.120<br>6.015 | 2.420<br>2.715 | 40.06<br>40.33 | 7.77<br>7.47 |
| Protein of soy bean | 4.011<br>4.112 | 6.000<br>6.115 | 2.790<br>2.880 | 40.78<br>40.55 | 7.78<br>7.84 |
| Calc. for $C_5H_{11}SNO_2$ | — | — | — | 40.23 | 7.43 |

Table XXII *Analysis for Nitrogen of the amino acid.*

| Nitrogen / Sample protein | Sample taken (mg) | $N_2$ (cc) | Temp. (°C) | Atmos. press. (mm.) | N % |
|---|---|---|---|---|---|
| Muscle protein of sardine | 3.600<br>4.435 | 0.292<br>0.356 | 29.5<br>28.5 | 760.0<br>761.0 | 9.15<br>9.10 |
| Muscle protein of whale | 5.380<br>3.300 | 0.448<br>0.281 | 29.3<br>30.0 | 759.5<br>759.0 | 9.39<br>9.60 |
| Protein of silk-worm pupa | 2.630<br>4.310 | 0.216<br>0.358 | 29.0<br>29.5 | 759.8<br>760.0 | 9.27<br>9.38 |
| Protein of soy bean | 6.345<br>3.495 | 0.524<br>0.299 | 29.5<br>30.5 | 760.5<br>760.0 | 9.34<br>9.69 |
| Casein | 4.527<br>3.795 | 0.369<br>0.314 | 29.0<br>29.0 | 759.0<br>759.0 | 9.16<br>9.30 |
| Calc. for $C_5H_{11}SNO_2$ | — | — | — | — | 9.39 |

It is clearly seen from the above results that the isolated crystals are composed in the main part of methionine, though contaminated with a trace of impurity.

B. *Derivatives of methionine.*

(a) *Copper salt:* This salt crystallizes out soon after the saturated solution of copper acetate is added to the concentrated aqueous solution of the free amino acid.

(b) *α-Naphthylisocyanate derivative:* 0.12 cc of α-naphthylisocyanate (Kahlbaum) is added to a solution of 0.1 g of the free amino acid dissolved in 6.7 cc of $N/10$ Na OH, and shaken for 2 hours. On acidifying the filtrate of the reaction mixture, a mass of white needle-like crystal forms immediately. The mixture is centrifuged to separate the crystal, which is washed several times with water, and dried to a constant weight at 80 ~ 90°C. The product melted at 183 ~ 186°C, but on recrystallizing from hot 70 per cent alcohol it melted at 190 ~ 193°C. The yield of the first crystals of these two derivatives is shown in Table XXIII.

TABLE XXIII *Yield of Copper Salt and α-Naphtylisocyanate.*

| | Copper salt (g) | α-Naphthyl-isocyanate (g) |
|---|---|---|
| 0.1 g crystal from muscle protein of sardine | 0.09 | 0.16 |
| 0.1 g 〃 〃 〃 〃 〃 whale | 0.084 | 0.19 |
| 0.1 g 〃 〃 the protein of silk-worm pupa | 0.09 | 0.19 |
| 0.1 g 〃 〃 〃 〃 〃 soy bean | — | — |
| 0.05 g 〃 〃 〃 〃 〃 〃 〃 | 0.015 | — |
| 0.1 g 〃 〃 casein | 0.07 | 0.18 |

The two derivatives thus prepared were analyzed to determine the content of nitrogen, copper and sulphur. The results are given in Tables XXIV ~ XXVII. The analytical data of these two derivatives of the amino acid isolated from five kinds of protein agree well with the theoretical value of methionine.

## IV. Contamination of Methionine Fraction with Glutamic Acid

In most cases, methionine was isolated in relatively pure crystal by the method adopted; but in the case of protein of silk-worm pupa, it was found that the isolated fraction was largely contaminated with glutamic acid. This impurity was removed and identified by its nitrogen content and its acidity. The removal of the impurity is as follows: The mixed crystals were dissolved in a small amount of

TABLE XXIV *Analysis for Nitrogen of Methionine-Copper Salt.*

| Nitrogen / Sample protein | Sample taken (mg) | $N_2$ (cc) | Temp. (°C) | Atmos. press. (mm.) | N % |
|---|---|---|---|---|---|
| Muscle protein of sardine | 4.700 | 0.323 | 24.5 | 751.5 | 7.80 |
| Muscle protein of whale | 4.215 | 0.300 | 24.5 | 752.5 | 7.92 |
| Protein of silk-worm pupa | 4.710 | 0.320 | 24.5 | 751.0 | 7.72 |
| Protein of soy bean | 4.832 | 0.338 | 24.5 | 752.5 | 7.66 |
| Casein | 4.492 | 0.328 | 25.5 | 760.0 | 8.11 |
| Calc. for $Cu(C_5H_{10}SNO_2)_2$ | — | — | — | — | 7.79 |

TABLE XXV *Analysis for Copper of Methionine-Copper Salt.*

| Copper / Sample protein | Sample taken (mg) | CuO (mg) | Cu (mg) | Cu % |
|---|---|---|---|---|
| Muscle protein of sardine | 10.815 | 2.395 | 1.914 | 17.69 |
| Muscle protein of whale | 10.670 | 2.350 | 1.886 | 17.67 |
| Protein of silk-worm pupa | 11.155 | 2.505 | 2.002 | 17.94 |
| Protein of soy bean | 8.230 | 1.820 | 1.454 | 17.66 |
| Casein | 9.175 | 2.021 | 1.615 | 17.60 |
| Calc. for $Cu(C_5H_{10}SNO_2)_2$ | — | — | — | 17.66 |

TABLE XXVI *Analysis for Nitrogen of α-Naphtylisocyanate.*

| Nitrogen / Sample protein | Sample taken (mg) | $N_2$ (cc) | Temp. (°C) | Atmos. press. (mm.) | N % |
|---|---|---|---|---|---|
| Muscle protein of sardine | 4.184 | 0.331 | 30.5 | 755.3 | 8.84 |
| Muscle protein of whale | 5.085 | 0.397 | 30.0 | 756.0 | 8.75 |
| Protein of silk-worm pupa | 4.693 | 0.372 | 30.5 | 755.0 | 8.87 |
| Protein of soy bean | 5.113 | 0.407 | 28.0 | 745.0 | 8.96 |
| Casein | 3.688 | 0.285 | 25.0 | 749.5 | 8.65 |
| Calc. for $C_{16}H_{18}SN_2O_3$ | — | — | — | — | 8.80 |

TABLE XXVII *Analysis for Sulphur of α Naphtylisocyanate.*

| Sulphur / Sample protein | Sample taken (mg) | $BaSO_4$ (mg) | S % |
|---|---|---|---|
| Muscle protein of sardine | 10.519 | 7.935 | 10.36 |
| Muscle protein of whale | 10.690 | 7.850 | 10.11 |
| Protein of silk-worm pupa | 11.425 | 8.419 | 10.12 |
| Protein of soy bean | 11.235 | 8.412 | 10.28 |
| Casein | 10.475 | 7.840 | 10.28 |
| Calc. for $C_{16}H_{18}SN_2O_3$ | — | — | 10.08 |

water, and methionine was precipitated by mercuric chloride; from the filtrate, excess of the reagent was removed by hydrogen sulfide, the hydrochloric acid produced was eliminated by silver oxide, the excess of which was removed by hydrogen sulfide; upon concentrating the filtrate, crystal amounting to 0.25 per cent of the protein of silk-worm pupa was obtained. This crystal melted at 220°C. As is shown in Table XXVIII, the results of determination of its acidity and of its nitrogen by both the micro DUMAS method and the VAN SLYKE method, indicate this crystal to be glutamic acid.

TABLE XXVIII *Identification of Contaminated Crystal.*

| Nitrogen content / Method | Sample taken (mg) | $N_2$ (cc) | Temp. (°C) | Atmos. press. (mm.) | N % |
|---|---|---|---|---|---|
| Micro DUMAS | 3.820 | 0.324 | 20.5 | 768.0 | 9.97 |
| VAN SLYKE | 1.00 | 0.17 | 23.0 | 769.0 | 9.65 |
| Calc. for $C_5H_9NO_4$ | — | — | — | — | 9.52 |

| | Sample taken (mg) | cc $N/45$ NaOH required for neutralization | Calc. for glutamic acid from the titre (mg) |
|---|---|---|---|
| Acidity | 5.00 | 1.30 | 4.24 |
| | 6.53 | 1.86 | 6.08 |

Furthermore, some crystal giving strong millon reaction was obtained in the course of isolating methionine from casein hydrolyzate, viz., upon concentrating, in vacuo, the solution which was obtained after the removal of mercury by hydrogen sulfide from methionine-sublimate precipitate. This crystal melted and decomposed at 302°C, and the analytical result showed that this was tyrosine.

4.845 mg subst.: 0.332 cc $N_2$ (at 22°, 767 mm)

| | |
|---|---|
| Found | 7.81 % |
| Calc. for $C_9H_{11}NO_3$ | 7.74 % |

## Summary

1. Methionine in crystalline form was isolated from the muscle protein of sardine and whale, the protein of silk-worm pupa, the protein of soy bean, and casein. The yield based on dry and ash-free protein was as follows:

| | |
|---|---|
| Muscle protein of sardine | 0.52 % |
| Muscle protein of whale | 0.37 % |
| Protein of silk-worm pupa | 0.43 % |
| Protein of soy bean | 0.08 % |
| Casein | 0.41 % |

2. Contrary to the result of ODAKÉ, methionine was isolated with certainty, though in a small amount from the protein of soy bean.

3. Although only a few kinds of protein were investigated in the present study, it might be presumed that methionine would be generally wide-spread in proteins, especially in those of animal origin.

4. The methionine crystal isolated from silk-worm pupa was largely contaminated with glutamic acid. In the case of casein, tyrosine was isolated from the methionine fraction precipitated by sublimate.

## CHAPTER VII

## The Contents of Cystine and Methionine in Several Proteins

Having isolated methionine in crystalline form from several proteins, it has been shown that the protein of soy bean contained a far smaller amount of methionine than did either protein of silk-worm pupa or that of sardine. As

regards the contents of the other indispensable amino acids for nutrition, i.e., lysine-, histidine-, and tryptophane-content in these proteins have been shown to be practically the same except that the lysine content of soy bean protein was little lower than that of the other proteins.

The present work has been carried out in order to compare these proteins with one another as to their contents of sulphur containing amino acids, namely cystine and methionine. The rôle of cystine in nutrition can be replaced without fail by methionine,[57] [58] so that it is necessary to take the sum of these two sulphur containing amino acids as a criterion for the comparison of nutritional value of protein.

Now, if it is assumed that BÄRNSTEIN'S[56] statement is valid for every protein, that is, that the total sulphur in any protein consists exclusively of methionine-sulphur and cystine-sulphur, the methionine content can be calculated when cystine and total sulphur are determined. The total sulphur was determined by DENIS-BENEDICT method.[59] The determination of cystine was carried out according to OKUDA'S iodine method.[60] These results are given in Table XXIX.

Table XXIX shows that the cystine content of the protein of soy bean is higher than that of the other protein, whereas the content of methionine is quite contrary to that of cystine in the protein of soy bean. Regarding the sulphur containing amino acids as a whole, only a little difference is found between the protein of soy bean and any of the others.

TABLE XXIX *Contents of Cystine and Methionine.*

| Sample protein \ % of dry matter | Total S | Cystine S | Difference | Methionine (1) | Methionine (2) | Cystine | Cystine + Methionine |
|---|---|---|---|---|---|---|---|
| Muscle protein of sardine | 0.878 | 0.203 | 0.675 | 0.52 | 3.14 | 0.76 | 3.90 |
| Muscle protein of whale | 0.866 | 0.238 | 0.628 | 0.37 | 2.92 | 0.90 | 3.82 |
| Protein of silk-worm pupa | 0.870 | 0.230 | 0.640 | 0.43 | 2.97 | 0.86 | 3.83 |
| Protein of soy bean | 0.702 | 0.280 | 0.422 | 0.08 | 1.96 | 1.05 | 3.01 |
| Casein | 0.637 | 0.091 | 0.546 | 0.41 | 2.54 | 0.34 | 2.88 |

(1) Isolated quantities.

(2) Methionine calculated according to the formula:

$$(\text{Total sulphur} - \text{Cystine-sulphur}) \times \frac{149.16}{32} = \text{Methionine}$$

## CHAPTER VIII

## The Rate of Liberation of Amino Acids and Ammonia in Tryptic Hydrolysis

It is well known that a certain amount of ammonia is always liberated in addition to amino acids in the course of tryptic hydrolysis of protein, and it is presumed that its liberation is due to the action of certain special enzyme contained in the trypsin.[61] [62]

The question whether the ammonia is liberated from the amide or whether it originated in the amino acids is not yet determined, but while the amino acids produced by digestion are absorbed and utilized, the ammonia being the last product in the process of metabolism, any nutritive value in it is inconceivable. For this reason, the velocity and the quantity of nitrogen liberation in these forms in the course of digestion of protein are considered to be factors in determining its nutritive value. Therefore, studies have been made of the rate of liberation of two compounds in the tryptic digestion of such proteins as that of sardine, silk-worm pupa, and soy bean.

### Method

(1) *Substrates:* The materials used for the present experiment were the same kinds of protein as were used in Chapter X.

(2) *Buffer solution:* The preliminary experiment was carried out in order to keep the pH of the reaction mixture constant. Both substrate and the enzyme together with some tricresol were added to either $M/10$ or $M/5$ boric acid buffer solutions (pH 8.6), and incubated for three days at 38°. In every case, it was observed that the pH of the reaction mixture was changed to one lower than 8.0. However, if a mixed solution of equal quantities of $M/5$ boric acid buffer solution and $1\,M$ $NaHCO_3$ was used, there was no change in its pH value for several days, therefore this solution was adopted for the experiment.

(3) *Enzyme:* In a preliminary experiment, permitting the trypsin of Kahlbaum and of Grübler act on casein at pH 8.6 and 38° for 20 hours, their activities were compared and it was found that the former activity was far greater than the latter one. Hence, the Kahlbaum trypsin was chosen for the experiment.

| | Kahlbaum-trypsin | | Grübler-trypsin | |
|---|---|---|---|---|
| | (+) | (−) | (+) | (−) |
| Per cent of hydrolysis | 30.6 | 20.3 | 14.5 | 14.7 |

(+) indicates the addition of enterokinase.

(−) indicates no addition of enterokinase.

(4) *Preparation of enterokinase:* The mucous membrane of the upper part (two meters or less) of the pig's small intestine was taken and dehydrated by acetone. Then it was treated with ether, and the powder thus obtained was extracted with *M*/10 boric acid buffer solution (pH 8.6) at room temperature for one hour; the insoluble part was removed by centrifuging, and the soluble part was used as the kinase. Then, the quantity of the enterokinase, which is required for fully activating the Kahlbaum trypsin, was determined as is shown in the following preliminary experiment.

The reaction components given in Table XXX, except casein, were mixed and the trypsin was thus activated by keeping it at 30° for one hour; then 2% casein solution (pH 8.6) was added and well mixed. One hour later, in order to stop the reaction as well as to remove the protein, sulphosalicylic acid was added and filtered. The amino-N in the filtrate was estimated by VAN SLYKE method. The results are given in Table XXX.

TABLE XXX *Amount of the Kinase for activating the trypsin.*

| *M*/10 Boric acid buffer solution pH 8.6 (cc) | $H_2O$ | Trypsin (cc) | Enterokinase (cc) | 2% Casein (cc) | Amino-N × 2 (cc) |
|---|---|---|---|---|---|
| 5 | 4.0 | 2 | 1.0 (0.02 g) | 5 | 2.7 |
| 〃 | 3.5 | 〃 | 1.5 | 5 | 2.8 |
| 〃 | 2.5 | 〃 | 2.5 | 5 | 3.0 |
| 〃 | 0.0 | 〃 | 5.0 | 5 | 3.0 |
| 〃 | 5.0 | 〃 | 0 | 5 | 2.5 |
| 〃 | 10.0 | 〃 | 0 | 0 | 1.1 |
| 〃 | 5.0 | 〃 | 5 | 0 | 1.1 |

It is clearly seen from Table XXX that 0.1 g of the trypsin used was completely activated by the solution which is equivalent to 0.05 g of the kinase powder, hence the trypsin solution activated in the foregoing way was used throughout the experiment.

(5) *Digestion experiment:* The reaction system was made up as in the following combination. For each of the proteins, the protein digest (main experiment) and the protein control were set in several flasks, using one flask for each digestion period, and at the same time was also made the enzyme control, which was common to all kinds of protein.

| | |
|---|---|
| I. The main experiment | 1 g of sample protein + 20 cc buffer solution + 2 cc activated trypsin. |
| II. Protein control | 1 g of sample protein + 20 cc buffer solution + 2 cc water. |
| III. Enzyme control | ——— 20 cc buffer solution + 2 cc activated trypsin. |

To each of these flasks, tricresol (Kahlbaum) up to 0.5 % was added and they were left in a thermostat at 38°C. After 2, 24, 73, and 163 hours, reaction was stopped by neutralizing with hydrochloric acid and then adding sulphosalicylic acid. The amino-N in the filtrate was determined by VAN SLYKE method, and ammonia-N by the aeration method according to VAN SLYKE and CULLEN.[63] In such a digestion condition as they were left at 38° and pH 8.6, it is supposed that the ammonia formed might escape more or less and therefore, in the preliminary experiment, the ammonia set free in the gaseous phase of the flask was caught continuously during the digestion. It was ascertained that the quantity of it was so small that it was within the limit of experimental error, and so in the digestion experiment the flasks were simply left stoppered tight.

## Experimental Results

The quantities of amino-N and ammonia-N liberated by protein digest and protein- and enzyme-control are given in Table XXXI. Subtracting both of the protein- and enzyme-control from the value of the corresponding protein digest, the net values of two forms of nitrogen due to the tryptic digestion only from each protein are given in Table XXXII, in which the values are expressed as per cent of the total nitrogen of each protein, as well as per cent of the amino-N and the ammonia-N yielded upon the complete hydrolysis of the corresponding protein by boiling with 20 % HCl. These values are graphically shown in Figs. 10, 11, 12, and 13.

TABLE XXXI

*Gross Results and Controls.*

| Hours of digestion | Protein digest | | | | Protein control | | | | Enzyme control | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Amount of N as | | % of total N as | | Amount of N as | | % of total N as | | Amount of N as | |
| | $-NH_2$ | $NH_3$ | $-NH_2$ | $NH_3$ | $-NH_2$ | $NH_3$ | $-NH_2$ | $NH_3$ | $-NH_2$ | $NH_3$ |
| A. Protein of sardine (Total N of digest 164.26 mg, of substrate 150.22 mg). | | | | | | | | | | |
| 2 | 12.79 | 0.566 | 7.78 | 0.34 | 0.544 | 0.198 | 0.33 | 0.12 | 5.034 | 0.0804 |
| 24 | 33.87 | 1.509 | 20.62 | 0.92 | 0.731 | 0.321 | 0.44 | 0.19 | 6.668 | 0.3459 |
| 73 | 41.69 | 2.255 | 22.64 | 1.19 | 0.968 | 0.569 | 0.59 | 0.35 | 7.388 | 0.4545 |
| 163 | 47.69 | 3.127 | 29.04 | 1.90 | 1.374 | 0.914 | 0.84 | 0.56 | 7.600 | 0.4695 |
| Complete Hydrolysis | 111.32 | 8.425 | 74.10 | 5.61 | | | | | | |
| B. Protein of soy bean (Total N of digest 141.79 mg, of substrate 127.75 mg). | | | | | | | | | | |
| 2 | 9.324 | 0.701 | 6.58 | 0.49 | 0.604 | 0.321 | 0.43 | 0.23 | | |
| 24 | 27.56 | 1.860 | 19.43 | 1.31 | 1.157 | 0.420 | 0.82 | 0.29 | | |
| 73 | 33.48 | 3.238 | 23.61 | 2.28 | 1.218 | 0.642 | 0.86 | 0.45 | | |
| 163 | 40.55 | 4.043 | 28.59 | 2.85 | 1.378 | 1.063 | 0.97 | 0.74 | | |
| Complete Hydrolysis | 95.41 | 14.04 | 74.68 | 10.99 | | | | | | |
| C. Protein of silk-worm pupa (Total N of digest 146.01 mg, of substrate 131.97 mg). | | | | | | | | | | |
| 2 | 9.804 | 0.404 | 6.71 | 0.28 | 0.785 | 0.321 | 0.54 | 0.22 | | |
| 24 | 22.52 | 1.051 | 15.43 | 0.72 | 0.973 | 0.420 | 0.67 | 0.29 | | |
| 73 | 29.76 | 1.725 | 20.38 | 1.18 | 1.096 | 0.469 | 0.75 | 0.32 | | |
| 163 | 32.32 | 2.318 | 22.14 | 1.59 | 1.553 | 0.865 | 1.06 | 0.59 | | |
| Complete Hydrolysis | 98.01 | 13.83 | 74.27 | 10.48 | | | | | | |

TABLE XXXII

*Net Results of Tryptic Digestion.*

| Hours of digestion | Amount of nitrogen as | | Per cent of total N as | | Per cent of total hydrolysis | |
|---|---|---|---|---|---|---|
| | $-NH_2$ | $NH_3$ | $-NH_2$ | $NH_3$ | $-NH_2$ | $NH_3$ |
| | | | Protein of sardine. | | | |
| 2 | 7.209 | 0.288 | 4.79 | 0.19 | 6.47 | 3.42 |
| 24 | 26.467 | 0.842 | 17.62 | 0.56 | 23.78 | 9.99 |
| 73 | 33.333 | 1.232 | 22.19 | 0.82 | 29.92 | 14.62 |
| 163 | 38.719 | 1.743 | 25.78 | 0.16 | 34.78 | 20.69 |
| | | | Protein of soy bean. | | | |
| 2 | 3.686 | 0.299 | 2.88 | 0.23 | 3.86 | 2.13 |
| 24 | 19.731 | 1.094 | 15.44 | 0.86 | 20.68 | 7.79 |
| 73 | 24.869 | 2.141 | 19.47 | 1.51 | 26.07 | 15.25 |
| 163 | 31.569 | 2.511 | 24.71 | 1.97 | 33.09 | 17.89 |
| | | | Protein of silk-worm pupa. | | | |
| 2 | 3.984 | 0.0028 | 3.02 | 0.002 | 4.06 | 0.02 |
| 24 | 14.885 | 0.285 | 11.28 | 0.22 | 15.19 | 2.06 |
| 73 | 21.272 | 0.801 | 16.12 | 0.61 | 21.70 | 5.79 |
| 163 | 23.166 | 0.984 | 17.55 | 0.75 | 23.64 | 7.12 |

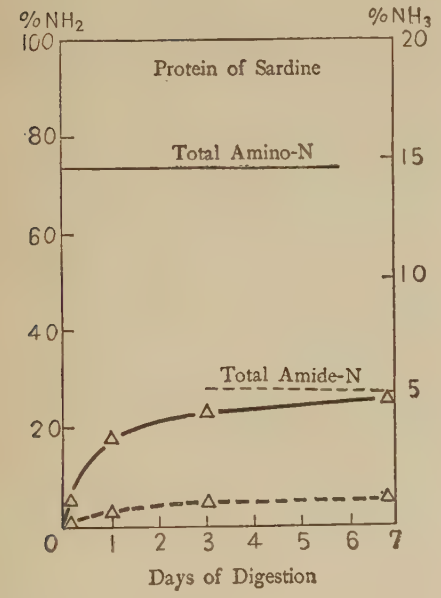


Fig. 10.

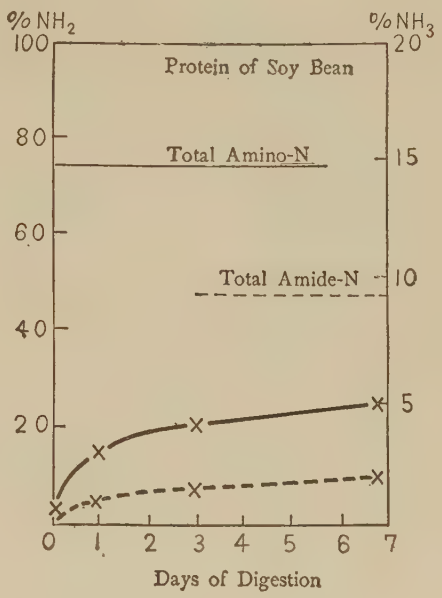


Fig. 11.

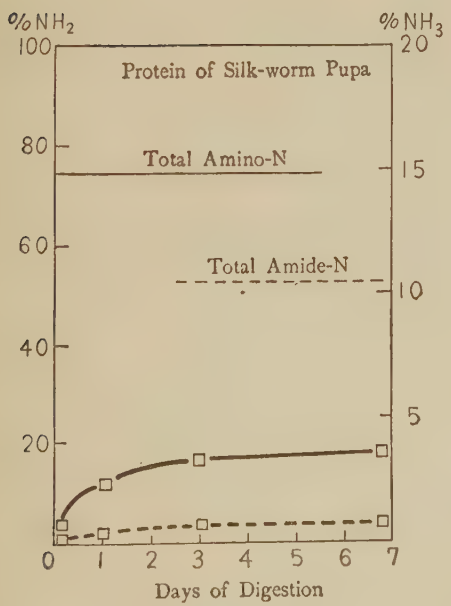


Fig. 12.

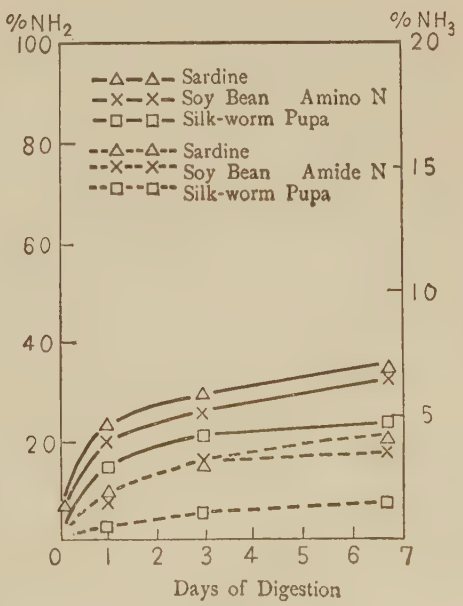


Fig. 13.

When the process of trypic digestion is treated as monomolecular reaction, the results are as shown in Table XXXIII and illustrated in Fig. 14.

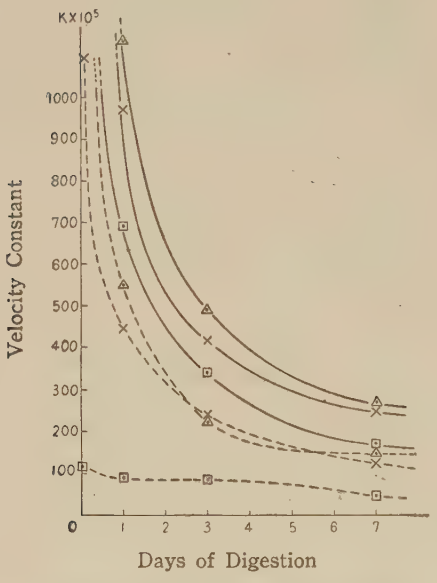


FIG. 14. The change of the velocity constant with the digestion

As is general in the case of enzyme reaction, the velocity constant of liberation of amino-N and ammonia-N from each protein by trypsin showed markedly greater value at the first stage and decreased as time elapsed.

Of these three proteins, the velocity of liberation of amino-N is greatest in sardine, and smaller in the cases of soy bean and silk-worm pupa, respectively. Under the reacting condition above-mentioned, the degree of hydrolysis attained after 7 days' digestion, expressed as per cent of complete hydrolysis brought about by boiling with 20 % HCl, was 35 for sardine, 33 for soy bean, and 24 for silk-worm pupa. In the former two, the increasing tendencies of digestion even after 7 days were observed, while in silk-worm pupa the reaction almost stopped.

In all three cases, the velocity constant of the liberation of ammonia was smaller than that of the amino acid. Remarkably enough, there was not much difference between the proteins of sardine and soy bean, while the velocity constant for the protein of silk-worm pupa was far smaller than that of the former two. In regard to the quantity of ammonia yielded by the equal quantities of the three kinds of protein, the soy bean yielded the largest quantity, while the sardine yielded the smallest; this may be fully accounted for by the law of mass action.

Although three proteins were digested by the trypsin in the foregoing experiment, a certain amount of erepsin must have been contained in the preparation of enterokinase, and hence it should be taken into account that the latter might also have participated more or less in the digestive action.

## Summary

1. The proteins of sardine, silk-worm pupa, and soy bean were hydrolyzed at

TABLE XXXIII

| $t$ (hours) | Amino-N | | | | $t$ (hours) | Ammonia-N | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Amino-N (mg) | $x$ (mg) | $a-x$ (mg) | $K=1/t\cdot\ln a/(a-x)$ | | Ammonia-N (mg) | $x$ (mg) | $a-x$ (mg) | $K=1/t\cdot\ln a/(a-x)$ |
| | | | $a=110.771$ | Protein of sardine | | $a=8.227$ | | | |
| 0 | 0.544 | | | | 0 | 0.198 | | | |
| 2 | 7.743 | 7.209 | 103.562 | 0.03366 | 2 | 0.486 | 0.288 | 7.939 | 0.01781 |
| 24 | 27.011 | 26.467 | 84.304 | 0.01138 | 24 | 1.040 | 0.842 | 7.385 | 0.00450 |
| 73 | 33.866 | 33.333 | 77.438 | 0.00490 | 73 | 1.430 | 1.232 | 6.995 | 0.00222 |
| 163 | 39.263 | 38.719 | 72.052 | 0.00264 | 163 | 1.941 | 1.743 | 6.484 | 0.00146 |
| | | | $a=94.806$ | Protein of soy bean | | $a=13.718$ | | | |
| 0 | 0.604 | | | | 0 | 0.321 | | | |
| 2 | 4.290 | 3.686 | 91.120 | 0.01982 | 2 | 0.720 | 0.299 | 13.419 | 0.01098 |
| 24 | 20.335 | 19.731 | 75.075 | 0.00972 | 24 | 1.415 | 1.094 | 12.624 | 0.00348 |
| 73 | 25.473 | 24.869 | 69.937 | 0.00416 | 73 | 2.462 | 2.141 | 11.577 | 0.00237 |
| 163 | 32.173 | 32.569 | 63.237 | 0.00248 | 163 | 2.832 | 2.511 | 11.207 | 0.00124 |
| | | | $a=97.225$ | Protein of silk-worm pupa | | $a=13.506$ | | | |
| 0 | 0.895 | | | | 0 | 0.321 | | | |
| 2 | 4.769 | 3.984 | 93.241 | 0.02087 | 2 | 0.324 | 0.003 | 13.503 | 0.00115 |
| 24 | 15.670 | 14.885 | 82.340 | 0.00692 | 24 | 0.606 | 0.285 | 13.221 | 0.00089 |
| 73 | 22.057 | 21.272 | 75.953 | 0.00338 | 73 | 1.122 | 0.801 | 12.705 | 0.00084 |
| 163 | 23.951 | 23.166 | 74.059 | 0.00167 | 163 | 1.305 | 0.984 | 12.522 | 0.00046 |

$x$ = quantity of amino-N or ammonia-N liberated.

$a$ = quantity of amino-N or ammonia-N yielded upon the complete hydrolysis by 20 per cent HCl.

38°C and pH 8.6 by the Kahlbaum's trypsin completely activated by enterokinase, and both the amino-N and the ammonia-N liberated during digestion were determined after 2, 24, 73, and 163 hours, and the velocities of liberation of these two forms of nitrogen were compared.

2. The reactions of liberation of amino-N and ammonia-N were not conformable to the monomolecular reaction. In the initial stage of the digestion, the velocity constant treated as a monomolecular reaction showed markedly greater values and as the digestion progressed their values became gradually smaller. In the case of the proteins of sardine and of soy bean, digestion continued more or less even after seven days, while, in the case of the protein of silk-worm pupa, equilibrium was already noticed after three days' digestion.

3. The quantity of amino-N liberated, expressed as to per cent, both of total nitrogen and of total amino-N, was greatest in the protein of sardine, and it was far less in the proteins of soy bean and silk-worm pupa. The quantity of the ammonia-N liberated, expressed as per cent of total nitrogen of each of the proteins, was greatest in the protein of soy bean, and there was hardly any difference between that of silk-worm pupa and sardine, but if it is expressed as per cent of the amide-N contained in the protein, it is practically the same in the proteins of soy bean and of sardine, while in the protein of silk-worm pupa it was much smaller than in those of the former two.

## CHAPTER IX

### A Comparative Study of the Methods for Isolating Diamino Acids

Since the well-known silver-baryta method was first devised by KOSSEL,[64] it has been extensively applied to the isolation of the diamino acids, histidine, arginine, and lysine. CALVERY,[65] and VICKERY and coworkers[66] modified the KOSSEL's method to a certain extent. Recently SASAKI[67] also modified the method on the basis of experimental results obtained by VICKERY and LEAVENWORTH,[66] CALVERY,[66] VICKERY and BLOCK,[66] and VICKERY and SHORE.[66] He analyzed, according to his modified method, the total coagulable protein part of soy bean, and obtained the yields of the diamino acids which approximate the values calculated from the results of nitrogen distribution according to the VAN SLYKE method. The present chapter

deals with the comparison of the original KOSSEL's method with the SASAKI's method in the isolation of the diamino acids from the protein of sardine.

## I. The Isolation of the Diamino Acids by the Original Kossel Method

From 100 g of the protein of sardine (moisture 2.85 %, ash 1.62 %, total nitrogen 14.90 %), the diamino acids have been isolated by slightly modified KOSSEL's method. The procedure for the isolation may be outlined as follows.

The sample was hydrolyzed by boiling with 33 % sulfuric acid until the hydrolyzate showed the negative biuret reaction. An excess of sulfuric acid was removed by baryta, and ammonia was distilled off under reduced pressure in the presence of a slight excess of baryta. The silver salt of histidine and arginine was precipitated by adding an excess of silver nitrate and baryta.

The precipitate, suspended in dilute sulfuric acid, was decomposed by hydrogen sulfide. After evaporating the filtrate, purine base was removed by adding an excess of silver nitrate. The filtrate, containing histidine and arginine, was neutralized by baryta and, in concentrating it with barium carbonate, the silver salt of histidine was precipitated. The silver salt was converted into the mercury salt and further into the dihydrochloride. The yield of the histidine dihydrochloride was 1.4 g.

The filtrate, containing arginine, from the precipitate of the silver salt of histidine was saturated with baryta, and the silver salt of arginine thus obtained was filtered off. The silver salt was suspended in water containing a small amount of sulfuric acid and decomposed with hydrogen sulfide. The silver sulfide was filtered off and the arginine was precipitated as the monopicrate, the yield of which was 5.9 g. The filtrate from the picrate was freed from picric acid and the remainder of arginine was precipitated with flavianic acid. 4.2 g of the arginine flavianate were obtained.

The filtrate, containing lysine, from the silver salt of histidine and arginine was freed from silver and barium ion, and then lysine was precipitated, in the presence of 5 per cent sulfuric acid, as phosphotungstate. The precipitate was dissolved in a mixture of one part of water and three parts of acetone, and the solution was freed from phosphotungstic acid by means of baryta, an excess of the latter being removed by sulfuric acid. The lysine was isolated as a monopicrate, the yield being 13.5 g.

The yields of the isolated derivatives of the diamino acids and the identifications of these derivatives are given in Tables XXXIV and XXXV, respectively.

## II. The Isolation of the Diamino Acids by the Sasaki's Method

The same protein sample aforementioned was subjected to the isolation of the diamino acids according to the Sasaki's procedure with a slight modification, which may be outlined as follows.

100 g of the protein sample were hydrolyzed with 20 per cent hydrochloric acid. After the glutamic acid hydrochloride (3.4 g) had been removed by the saturation with hydrochloric acid gas, most of the hydrochloric acid was removed by evaporating under reduced pressure and the remained hydrochloric acid was removed as silver chloride. A large excess of silver oxide was added, keeping the pH of the solution at $3 \sim 4$ by the addition of sulfuric acid. Thus, in the presence of an excess of silver ion, histidine silver salt was precipitated by adding baryta until the pH of the solution came to $7 \sim 7.2$. From the filtrate, arginine silver salt was then precipitated by adjusting the pH of the filtrate at $11 \sim 12$.

The histidine silver salt was decomposed by sulfuric acid and hydrogen sulfide into histidine sulfate which was further purified by converting it into the mercury salt by Hopkins reagent. The mercury salt of histidine was decomposed by sulfuric acid and hydrogen sulfide and the freed histidine sulfate was precipitated as the picrate, the yield of which was 1.4 g. The filtrate from the picrate was freed from the picric acid, then treated with flavianic acid, but no flavianate was obtained.

The silver salt of arginine was decomposed by sulfuric acid and hydrogen sulfide, as usual. The filtrate containing arginine sulfate was treated with picric acid. The filtrate from the picrate, after being freed from an excess of picric acid, was further treated with flavianic acid. The picrate and monoflavianate of arginine totalled 2.3 g and 4.5 g, respectively.

The filtrate, containing lysine, from the silver salt of histidine and arginine was freed from silver and barium ion, and was precipitated by phosphotungstic acid as in the usual way. The precipitate was dissolved in a mixture of acetone and water, and decomposed by baryta. The freed lysine was precipitated by picric acid. 8.1 g of the crystalline picrate and a bulk of an amorphous picrate were obtained. The latter picrate, after being freed from picric acid, was again fractionated by converting it into silver salt. The crystalline picrate obtained from this second fractionation was 1.5 g of the lysine picrate and 0.8 g of the histidine picrate.

The yields of these derivatives of the diamino acids and the identification of them by analysis are given in Tables XXXVI and XXXVII.

Table XXXIV *Yields by Kossel Method.*

| Isolated derivatives | Amount of the derivatives isolated from 100 g of the protein | Amount of the amino acid calculated from the isolated derivatives | |
|---|---|---|---|
| Histidine dihydrochloride | 1.4 | 0.95 | |
| Arginine monopicrate | 5.9 | 2.34 | 3.84 |
| Arginine flavianate | 4.2 | 1.50 | |
| Lysine monopicrate | 13.5 | 5.25 | |

Table XXXV *Analysis of the Derivatives Isolated by Kossel Method.*

| Isolated derivatives | m. p. | | N % | | Picric acid % | | Cl % | |
|---|---|---|---|---|---|---|---|---|
| | exp. | theor. | exp. | theor. | exp. | theor. | exp. | theor. |
| Histidine dihydrochloride | 238–240° | 245° | 18.74 | 18.43 | | | 30.42 | 31.11 |
| Arginine monopicrate | 205° | 217–218° | 21.95 | 22.31 | 53.50 | 52.17 | S % | |
| Arginine flavianate | 245–250° | | | | | | 6.75 | 6.53 |
| Lysine monopicrate | 240–245° | 252° | 18.41 | 18.66 | 61.90 | 61.06 | | |

Table XXXVI *Yields by Sasaki Method.*

| Isolated derivatives | Amount of the derivatives isolated from 100 g of the protein | Amount of the amino acid calculated from the isolated derivatives | |
|---|---|---|---|
| Histidine dipicrate | 2.2 | 0.55 | |
| Arginine monopicrate | 2.3 | 0.91 | 2.51 |
| Arginine flavianate | 4.5 | 1.60 | |
| Lysine monopicrate | 9.6 | 2.60 | |

Table XXXVII *Analysis of the Derivatives Isolated by Sasaki Method.*

| Isolated derivatives | m. p. | | N % | | Picric acid % | | S % | |
|---|---|---|---|---|---|---|---|---|
| | exp. | theor. | exp. | theor. | exp. | theor. | exp. | theor. |
| Histidine dipicrate | 85° | 85–90° | 19.60 | 19.41 | 71.80 | 70.56 | | |
| Arginine monopicrate | 206° | 217–218° | 22.10 | 22.31 | | | | |
| Arginine flavianate | | | | | | | 6.80 | 6.53 |
| Lysine monopicrate | 243–247° | 252° | 18.52 | 18.66 | 61.82 | 61.05 | | |

## III. The Discussion of the Results

Tables XXXIV ~ XXXVII show that the original KOSSEL's method seems to be rather superior to SASAKI's method. SASAKI's method gave a lesser yield of lysine. This is apparently due to the formation of an amorphous form of picrate which was not obtained by the KOSSEL's method. On the other hand, the contents of diamino acids in the protein of sardine may be calculated from the values of the nitrogen distribution in the VAN SLYKE analysis. The following table shows the comparisons of the isolated quantities of the diamino acids with their contents in the protein calculated from the nitrogen distribution. This shows that the quantities

| | KOSSEL's method % | SASAKI's method % | Calculated values from VAN SLYKE analysis % |
|---|---|---|---|
| Histidine | 0.95 | 0.55 | 3.40 |
| Arginine | 3.84 | 2.51 | 5.45 |
| Lysine | 5.25 | 2.60 | 9.70 |

of diamino acids isolated by KOSSEL's method and SASAKI's method amount to 28 % and 16 % of the histidine-content, 70 % and 46 % of the arginine-content, and 54 % and 27 % of the lysine-content, respectively. From these results it may be mentioned that the amounts of diamino acids, which can be isolated by the silver-baryta method, will not depend so much upon the method of precipitation of diamino acids by silver and baryta, as upon the kind of acid which is used for crystallization. A further study, therefore, may be necessary of using organic acids other than picric acid which form the sparingly soluble and well crystallizable salts with these diamino acids.

**Summary**

1. A comparative study has been made of KOSSEL's method and SASAKI's method for determining diamino acids.

2. Judging from the yields of derivatives of diamino acid and their analytical values for nitrogen, the KOSSEL's method seems to be superior to the SASAKI's method.

3. In SASAKI's procedure, a difficulty was experienced in isolating lysine as picrate, since a quantity of an amorphous or resinous picrate formed.

4. The amounts of diamino acids isolated by these two methods from 100 g of the protein of sardine were as follows: 0.95 g histidine, 3.85 g arginine, and 5.25 g lysine by KOSSEL's method; 0.55 g histidine, 2.51 g arginine, and 2.60 g lysine by SASAKI's method.

## CHAPTER X

## On the Determination of Amino Acids by VAN SLYKE Method in the Proteins Containing Impurities

It is well known that the VAN SLYKE method is superior to any other method for determining certain amino acids in proteins, since it is feasible to determine the arginine, histidine, lysine and a part of the cystine content with a small quantity of protein. It has been noted, however, that, if the sample for the estimation is not a pure protein, noticeable difficulties arise from various impurities contained in the sample. Consequently, the method is not applicable without modification to the analysis of foods or feedstuffs. The isolation of the total protein in a pure state from the sample is necessarily required before subjecting the sample to the VAN SLYKE method. There is, however, no perfect method for the isolation of pure protein without loss from the sample. Even if a part of the protein be isolated in a pure state and analyzed, the analytical results do not show the contents of the amino acids in the sample protein as a whole. Accordingly, the method of analyzing the total protein part of the sample is to be examined.

GRINDLEY and his co-workers[68] [69] analyzed feedstuffs without removing any interfering substance. NOLLAU[70] subjected the feedstuffs to the analysis after removing ether soluble matters. HAMILTON, NEVENS, and GRINDLEY[71] attempted to remove from the sample various kinds of interfering substances, but their method involved rather complicated procedures which are tedious and may cause some errors.

First, experiments have been carried out to see the effects of the various kinds of interfering substances upon the VAN SLYKE method. Then, attempts have been made to devise an appropriate method for preparing sample proteins which contain the least amounts of the interfering substances. Soy bean, silk-worm pupa, and sardine have been used for the experiment, and the results have been compared with those given in the literature.

### Experimental Results and Discussions

#### I. EFFECTS OF ADDITION OF INTERFERING SUBSTANCES ON THE HYDROLYSIS OF PROTEIN

In order to ascertain the extent of errors arising from the presence of various kinds of substances, HART and SURE[72] hydrolyzed casein with glucose, sucrose, and

starch in quantity five times as much as casein, and obtained the results, summarized in Table XXXVIII. YANAGAWA and NISIKAWA[73] also examined the effects of various substances at 20 per cent level as shown in Table XXXIX.

TABLE XXXVIII

| Froms of nitrogen | Casein | Casein + glucose | Casein + sucrose | Casein + starch |
|---|---|---|---|---|
| Amide-N | 10.39 | 10.09 | 8.54 | 9.20 |
| Humin-N | 1.47 | 3.76 | 9.15 | 7.46 |
| Cystine-N | 0.19 | 0.19 | 0.19 | 0.19 |
| Arginine-N | 7.95 | 7.59 | 6.77 | 6.74 |
| Histidine-N | 5.95 | 7.31 | 7.65 | 7.30 |
| Lysine-N | 9.41 | 7.01 | 6.38 | 5.54 |
| Monoamino-N | 59.32 | 57.85 | 55.38 | 50.89 |
| Non amino-N | 7.85 | 8.89 | 9.28 | 13.76 |
| Sum of N | 102.52 | 102.71 | 103.38 | 101.10 |

TABLE XXXIX

| | Amide-N | Humin-N | Diamino-N | Mono-amino-N |
|---|---|---|---|---|
| Casein | 10.73 | 1.73 | 27.13 | 60.41 |
| 〃 + glucose | 10.32 | 7.52 | 29.13 | 53.13 |
| 〃 + sucrose | 10.33 | 3.17 | 28.20 | 58.30 |
| 〃 + starch | 10.80 | 2.65 | 29.26 | 57.29 |
| 〃 + rape oil | 10.29 | 1.92 | 27.17 | 60.62 |
| 〃 + cotton | 10.36 | 2.14 | 28.92 | 58.58 |

Several experiments have been carried out to confirm the results obtained by these authors. Casein was hydrolyzed with or without the addition of starch, sucrose, filter paper, olive oil, and agar-agar and the hydrolyzates were analyzed by the VAN SLYKE method. The results are given in Table XL. In Table XL it has been clearly demonstrated that the addition of carbohydrate considerably influenced the various forms of nitrogen, while the addition of fat was shown to have almost no effect. From Table XL it will be seen that the interfering substances cause chiefly a remarkable increase in humin-N and some decrease in arginine-N, giving rise to further errors in the distribution of the various forms of nitrogen.

TABLE XL *Effect of Interfering Substances on Hydrolysis of Casein.*

| | Casein (Merck) | Casein + starch | Casein + sucrose | Casein + Filter paper | Casein + Olive oil | Casein + Agar-agar |
|---|---|---|---|---|---|---|
| Total N | 15.61 | 15.62 | 15.62 | 15.63 | 15.63 | 16.09 |
| Amide-N | 1.57 | 1.54 | 1.54 | 1.61 | 1.51 | 1.75 |
| Humin-N | 0.20 | 0.80 | 0.98 | 0.86 | 0.32 | 0.92 |
| Arginine-N | 1.24 | 0.96 | 0.87 | 0.86 | 1.13 | 1.03 |
| Cystine-N | 0.04 | 0.06 | 0.03 | 0.05 | 0.04 | 0.09 |
| Lysine-N | 1.54 | 1.41 | 1.69 | 1.35 | 1.50 | 1.51 |
| Histidine-N | 0.78 | 0.80 | 0.47 | 0.85 | 1.08 | 0.57 |
| Monoamino-N | 8.24 | 8.35 | 8.05 | 7.97 | 8.23 | 8.26 |
| Non amino-N | 2.18 | 1.77 | 1.86 | 2.21 | 1.79 | 2.02 |
| Sum of N | 15.79 | 15.69 | 15.49 | 15.76 | 15.60 | 16.15 |
| Total N | 100 | 100 | 100 | 100 | 100 | 100 |
| Amide N | 10.06 | 9.86 | 9.86 | 10.30 | 9.66 | 10.88 |
| Humin-N | 1.28 | 5.12 | 6.27 | 5.50 | 2.05 | 5.72 |
| Arginine-N | 7.94 | 6.15 | 5.57 | 5.50 | 7.23 | 6.40 |
| Cystine-N | 0.26 | 0.38 | 0.19 | 0.32 | 0.26 | 0.56 |
| Lysine-N | 9.87 | 9.03 | 10.82 | 8.64 | 9.60 | 9.38 |
| Histidine-N | 5.00 | 5.12 | 3.01 | 5.44 | 6.91 | 3.54 |
| Monoamino-N | 52.79 | 53.46 | 51.54 | 50.99 | 52.66 | 51.34 |
| Non amino-N | 13.97 | 11.34 | 11.91 | 14.14 | 11.45 | 12.55 |
| Sum of N | 101.17 | 100.46 | 99.17 | 100.83 | 99.82 | 103.37 |

The errors may be removed, on one hand, by purifying the samples without a loss of protein, or by modifying, on the other hand, the analytical procedures in which remarkable errors will take place.

## II. The Method for the Preparation of a Sample as Free as Possible from the Interfering Substances

Of the interfering substances such as sugar, starch, and cellulose which have been examined in the preceeding experiment, none except cellulose is difficult to remove by simply boiling with water. It was determined, accordingly, to what extent the total crude protein might be recovered from the hot watery extract by adding dilute acetic acid. Experiments were carried out using soy bean, silk-worm pupa, and sardine.

(1) 30 g soy bean were soaked in water overnight, then ground by a mortar into paste. It was diluted with water and a sufficient amount of sodium hydroxide was added to neutralize its acidity. The suspension thus obtained was boiled and filtered through cloth. The residue was extracted three times with 7 % sodium chloride and further extracted with boiling water All the filtered extracts were combined and filled up to a liter. The total nitrogen content of 10 cc extract was 11.35 mg.

(2) Sardine freed from skin, bone, and viscera, was ground in a meat chopper. A fine pasty material was obtained after pressing out through a wire gauze. 50 g of the paste were diluted up to a liter. The total nitrogen content of 10 cc solution was 14.14 mg.

(3) 40 g of silk-worm pupa were ground by a mortar, and extracted with water. After filtering the extract through cloth, the insoluble residue was further ground and the extraction was repeated several times. The watery extract was filled to a liter. The total nitrogen content of 10 cc solution was 12.04 mg.

The total nitrogen, protein-nitrogen, and coagulable protein-nitrogen in each extract were determined. The protein-nitrogen was estimated by STUTZER's method. The coagulable protein-nitrogen was estimated in the following way. An aliquot of the extract was boiled, a small amount of salt and an appropriate quantity of 5 % acetic acid was added, and the solution was boiled further several minutes. After cooling, the coagulated part was separated and washed, and subjected to the determination of nitrogen. Table XLI shows that the coagulable protein-nitrogen amounts to from 91 to 95 per cent of the protein-nitrogen, in other words, the protein part which can be coagulated by boiling at the optimum flocculating point represents practically the total protein in the sample. Thus, by the above-

TALBE XLI *Distribution of Protein- and Coagulable Protein-N.*

| | Soy bean | | Sardine | | Silk-worm pupa | |
|---|---|---|---|---|---|---|
| Total N | 100 | | 100 | | 100 | |
| Protein-N | 90.0 | 100 | 89.6 | 100.0 | 92.5 | 100.0 |
| Coagulable protein-N | 84.4 | 93.8 | 85.4 | 95.3 | 83.8 | 90.6 |
| Soluble N | | 6.2 | | 4.7 | | 9.4 |

mentioned method, it was possible to isolate a sample of protein which was almost equal to the total protein in the sample and which was free from most of the interfering substances.

III. The Recovery of Amino Acids from the Humin Precipitate

It has been shown in the paragraph (1) that the interfering substances make for an increase of the humin-N of the protein. The increase may be partly due to a catalytic decomposition of amino acids as Roxas[74] has pointed out, while the increase may also be accounted for by the concentration of nitrogen due to an adsorptive property of humin. Attempts were made to recover amino acids from the humin precipitate. There forms in the van Slyke procedure two kinds of humin, the one the acid-insoluble and the other the alkali-insoluble, hence experiments were made separately on these two kinds of humin.

(a) The recovery from the alkali-insoluble humin.

The alkali-insoluble humin, which was obtained after the distillation of ammonia, was dissolved in dilute hydrochloric acid, boiled, and precipitated by neutralizing the acid. The nitrogen in the precipitate was determined. The difference of nitrogen in the precipitate before and after the treatment will show the amount adsorbed. Comparisons were made between the original method and the modified method by using egg albumin which was hydrolyzed in the presence of the interfering substances. From Table XLII, it will be seen that only a trace of nitrogen can be recovered from the alkali-insoluble humin.

(b) The effect of the removal of acid-insoluble humin.

To see whether or not the alkali-insoluble humin would be influenced by removing

Table XLII *The Recovery of Nitrogen from Humin.*

| | Total N | Alkali-insoluble humin-N | |
|---|---|---|---|
| | | Original method | Extraction method* |
| Egg albumin | 12.34 | 0.21 | — |
| 〃 + cellulose | 12.36 | 0.30 | 0.25 |
| 〃 + starch | 12.38 | 0.49 | 0.41 |
| 〃 + glucose | 12.32 | 0.49 | 0.42 |

* The extraction method involves the procedure by which the adsorbed nitrogen on humin is as much removed as possible.

the acid-insoluble humin, experiments were made on a hydrolyzate obtained by hydrolyzing egg albumin in the presence or absence of cellulose. Table XLIII indicates that the addition of cellulose to egg albumin does not increase the alkali-insoluble humin, but does increase the acid-insoluble humin. It will be concluded, accordingly, that the increase of humin-N is not due to the adsorption of amino acids by the precipitate, and that the acid-insoluble humin may be derived from the partial decomposition of certain amino acids in the presence of carbohydrate or allied compounds. Since in the VAN SLYKE method several forms of nitrogen are not obtained directly but by calculation, the increase of humin-N will influence certain other forms of nitrogen. Hence, if the procedure of the VAN SLYKE method would be modified in such a way that the acid insoluble humin be removed from the hydrolyzate, the decrease of humin-N by the removal will balance to some extent the decrease of certain other forms of nitrogen, i. e., arginine- and monoamino-N.

TABLE XLIII *The Effect of Cellulose on Humin-N.*

| | Acid insol.-N | Alkali-insoluble humin-N | |
|---|---|---|---|
| | | Original method | Extraction method* |
| Egg albumin | 0.12 | 0.07 | — |
| 〃 + cellulose | 0.37 | 0.06 | 0.05 |

## IV. COMPARISON OF THE VAN SLYKE METHOD WITH ITS MODIFIED METHODS

It has been ascertained that, with the exception of cellulose, the removal of interfering substances can be carried out without an appreciable loss of protein. Accordingly, the effects of cellulose on the VAN SLYKE method were compared with that on modified methods which included the HAMILTON method and another one in which the acid-insoluble humin was discarded before the analysis.

Table XLIV shows that in the presence of cellulose the original VAN SLYKE method gives higher values for humin- and nonamino-N than the control values without cellulose, and lower values for arginine- and monoamino-N than the corresponding controls. The modified method gives smaller values for humin- and histidine-N than the controls. On the whole, it will be concluded that there is no

* See foot-note on page 195.

choice between these methods. Hence it follows that it is not necessary to treat the sample with such a complex manner as HAMILTON recommends, if the analysis be made on the coagulable protein sample aforementioned which is free from most of the interfering substances.

TABLE XLIV *Comparisons of Several Modifications of van Slyke Method.*

| | Casein (Hammarsten) original VAN SLYKE method | Casein + Filter paper original VAN SLYKE method | Casein + Filter paper Acid insol. humin removed | Casein + Filter paper HAMILTON's method | Casein + Filter paper HAMILTON's method modified |
|---|---|---|---|---|---|
| Total N | 14.22 | 14.23 | 14.23 | 14.23 | 14.23 |
| Amide N | 1.53 | 1.50 | 1.51 | 1.58 | 1.49 |
| Humin-N | 0.18 | 0.46 | 0.07 | 0.21 | 0.22 |
| Arginine-N | 1 28 | 1.14 | 1.20 | 1.14 | 1.20 |
| Cystine-N | 0.12 | 0.12 | 0.10 | 0.10 | 0.10 |
| Lysine-N | 1.55 | 1.46 | 1.60 | 1.39 | 1.47 |
| Histidine-N | 0.61 | 0.65 | 0.48 | 0.83 | 0.77 |
| Monoamino-N | 8.19 | 8.03 | 8.03 | 8.06 | 8.15 |
| Non amino-N | 0.93 | 1.03 | 1.15 | 0.90 | 0.97 |
| Sum of N | 14.38 | 14.38 | 14.14 | 14.21 | 14.38 |
| 20% HCl sol. N | — | — | 14.00 | — | — |
| " " insol. N | — | — | 0.23 | — | — |
| Total N | 100 | 100 | 100 | 100 | 100 |
| Amide-N | 10.75 | 10.57 | 10.78 | 11.14 | 10.51 |
| Humin-N | 1 27 | 3.21 | 0.50 | 1.47 | 1.54 |
| Arginine-N | 9.02 | 7.99 | 8.57 | 8.00 | 8.43 |
| Cystine-N | 0.84 | 0.82 | 0.71 | 0.69 | 0.69 |
| Lysine-N | 10.89 | 10.26 | 11.43 | 9.78 | 10.36 |
| Histidine-N | 4.26 | 4.57 | 3.43 | 5.84 | 5.40 |
| Monoamino-N | 57.59 | 56.46 | 57.36 | 56.67 | 57.31 |
| Non amino-N | 6.57 | 7.24 | 8.22 | 6.35 | 6.85 |
| Sum of N | 101.19 | 101.12 | 101.00 | 99.94 | 101.09 |

## V. HYDROLYSIS OF THE PROTEINS OF SOY BEAN, SILK-WORM PUPA AND SARDINE

A number of studies have been done on the analysis of protein of soy bean. OSBORNE and CLAP[75] and JONES and WATERMANN[76] carried out the analysis of glycinin. FRIEDEMANN[77] worked on a protein which is soluble in 0.2% sodium

hydroxide. KIMURA[78] investigated the protein of soy bean cake, so called "Tofu". The total protein contained in soy bean was analyzed by NOLLAU,[70] GRINDLEY and SLATER,[69] MASUNO and NISIMURA,[79] SASAKI,[80] and HAMILTON, UYEI and BAKER.[81] These authors except SASAKI or HAMILTON analyzed fat-free soy bean or soy bean as it is. As regards the proteins of silk-worm pupa, TUTIMIKADO and KASAI[82] carried out the analysis of a protein which is soluble in dilute alkali, and ÔSHIMA[83] analyzed the total coagulable protein. Recently, IZUMÉ and his co-workers[84] made a VAN SLYKE analysis on a protein part which was prepared by boiling fat-free silk-worm pupa with 0.1 % trichloracetic acid. The protein of sardine flesh was investigated by SEKINÉ and AKIYAMA.[85]

In order to compare the composition of these three proteins, analyses were carried out on the sample proteins which were prepared according to the following method. Based on the results obtained in paragraph (1), the preparation of the sample was carried out as follows: the material, after being finely ground, was boiled with water acidified with acetic acid, thus the impurities, such as sugar, glycogen, starch, and non protein nitrogeneous compound being removed. The total protein was obtained after extracting the coagulated part with alcohol and ether to remove the lipoid. The three sample proteins thus obtained were analyzed after the original VAN SLYKE method, the correction being applied for the solubility of phosphotungstate. The results were shown in Table XLV.

TABLE XLV *Analysis of Coagulable Proteins of Soy bean, Silk-worm Pupa, and Sardine.*

| In 100 parts of moisture and ash-free protein \ Kinds of protein | Protein of soy bean | | Protein of silk-worm pupa | | Protein of sardine | |
|---|---|---|---|---|---|---|
| Total N | 14.80 | 100 | 14.20 | 100 | 15.67 | 100 |
| Amide-N | 1.54 | 10.39 | 1.53 | 10.77 | 1.14 | 7.28 |
| Humin-N | 0.34 | 2.32 | 0.52 | 3.69 | 0.38 | 2.45 |
| Arginine-N | 2.41 | 16.30 | 1.52 | 10.70 | 1.84 | 11.74 |
| Cystine-N | 0.22 | 1.50 | 0.16 | 1.09 | 0.15 | 0.96 |
| Lysine-N | 1.18 | 7.99 | 1.36 | 9.56 | 1.95 | 12.45 |
| Histidine-N | 0.71 | 4.81 | 0.93 | 6.56 | 0.97 | 6.17 |
| Monoamino-N | 7.83 | 52.95 | 7.71 | 54.27 | 8.79 | 56.08 |
| Non amnio-N | 0.40 | 2.60 | 0.51 | 3.57 | 0.39 | 2.50 |
| Sum of N | 14.63 | 98.86 | 14.24 | 100.21 | 15.61 | 99.63 |

TABLE XLVI

*Protein of soy bean.*

| Forms of N \ Investigator | NOLLAU | HAMILTON UYEI and BAKER | SASAKI | The present author |
|---|---|---|---|---|
| Amide-N | 12.97 | 9.38 | 11.2 | 10.39 |
| Humin-N | 3.69 | 2.87 | 3.7 | 2.32 |
| Arginine-N | 15.52 | 15.70 | 13.0 | 16.30 |
| Cystine-N | 1.52 | 1.46 | 0.7 | 1.50 |
| Lysine-N | 7.02 | 6.18 | 7.6 | 7.99 |
| Histidine-N | 2.60 | 5.60 | 4.9 | 4.81 |
| Monoamino-N | 48.76 | 48.23 | 53.2 | 52.95 |
| Non amino-N | 7.12 | 2.43 | 4.2 | 2.60 |
| Sum of N | 99.20 | 91.90 | 98.6 | 98.86 |

*Protein of silk-worm pupa.*

| Forms of N \ Investigator | ÔSHIMA | IZUMÉ, YOSIMARU and YOSIMARU | The present author |
|---|---|---|---|
| Amide-N | 7.09 | 9.02 | 10.77 |
| Humin-N | 2.30 | 2.67 | 3.69 |
| Arginine-N | 12.84 | 14.27 | 10.70 |
| Cystine-N | 0.47 | 1.02 | 1.09 |
| Lysine-N | 6.69 | 6.27 | 9.56 |
| Histidine-N | 5.95 | 12.77 | 6.56 |
| Monoamino-N | 56.76 | 49.02 | 54.27 |
| Non amino-N | 7.91 | 4.73 | 3.57 |
| Sum of N | 100.01 | 99.77 | 100.21 |

*Protein of sardine.*

| Forms of N \ Investigator | SEKINÉ and AKIYAMA | | The present author |
|---|---|---|---|
| | ♂ | ♀ | |
| Amide-N | 3.83 | 0.71 | 7.28 |
| Humin-N | 4.29 | 5.52 | 2.45 |
| Arginine-N | 14.17 | 12.86 | 11.74 |
| Cystine-N | 0.19 | 0.08 | 0.96 |
| Lysine-N | 10.65 | 7.36 | 12.45 |
| Histidine-N | 5.80 | 11.40 | 6.17 |
| Monoamino-N | 62.08 | 63.17 | 56.08 |
| Non amino-N | | | 2.50 |
| Sum of N | 101.01 | 101.10 | 99.63 |

In Table XLV, no remarkable difference in amino acid between these proteins is observed except arginine-, lysine-, and histidine-N. The proteins of animal origin, namely, those of silk-worm pupa and sardine, are rich in lysine- and histidine-N and poor in arginine-N, compared with the protein of vegetable origin, namely, that of soy bean. In Table XLVI, the above results were compared with the corresponding values which have been hitherto obtained by a number of authors.

Regarding the distribution of nitrogen in soy bean protein, the result obtained by the author is similar to that obtained by SASAKI[80] except for arginine- and cystine-N, and is also similar to HAMILTON'S[81] result except for lysine- and histidine-N. Comparing the present result with that by NOLLAU,[70] it will be understood that the higher values for humin- and non amino-N in NOLLAU'S result is to be attributed to the interfering substances. In the protein of silk-worm pupa, differences especially in diamino acid fraction are noticed between the results by various authors. This is supposed to be due to the difference of the sample in either kinds, sexes, or the season when a silk-worm metamorphoses into a pupa. In the case of the protein of sardine, SEKINÉ and AKIYAMA[85] found a noticeable difference in amino acid between the two sexes, and so comparison of the results is not pertinent without knowing the sex of the sample. It may be said, however, that the higher values for humin-N obtained by SEKINÉ and AKIYAMA may be due to the interfering substances.

## Summary

1. The effects of the various substances on the determination of amino acid by the VAN SLYKE method have been examined and found to be such that the contamination of carbohydrate interferes considerably with the values for humin- and arginine-N, and that the presence of fat influences the results to a negligibly small extent. Hence, the method can not be applied directly to the analysis of proteins of foods or feedstuffs.

2. If the sample contains cellulose, there is little or no difference between the original VAN SLYKE method and modified methods including HAMILTON method and an another one in which the acid insoluble humin is first removed from the hydrolyzate. It can be said that the HAMILTON method has no advantage over the original method if the sample be purified somewhat before the analysis.

3. It has been found that the proteins of soy bean, sardine, and silk-worm pupa can be coagulated to the extent of 91 ~ 95 per cent of the total protein by boiling the materials with water acidified with acetic acid.

4. The total coagulable proteins of soy bean, sardine, and silk-worm pupa have been prepared and analyzed by the VAN SLYKE method. It has been shown that the proteins of sardine and silk-worm pupa give a similar distribution of various forms of nitrogen, but the protein of soy bean, compared with these two proteins, gives higher value for arginine-N and lower values for lysine- and histidine-N.

## RESUMÉ

*The first part of the dissertation can be summarized under the following conclusions.*

(1) The apparent dissociation constants for canavanine and canaline were as follows: $pK'_{a}$, $pK'_{b1}$, and $pK'_{b2}$ for canavanine were 9.25, 7.40, and 11.45, respectively; these values for canaline were 9.15, 10.30, and 11.55, respectively. The free energy change $\Delta F'_{298}$ due to the ionization of these groups has been calculated and found to be as follows: in canavanine, 12,600 calories for the carboxyl group, 10,100 calories for the guanidoxy group, and 15,700 calories for the $\alpha$-amino group; in canaline, 12,500 calories for the carboxyl group, 14,000 calories for the hydroxyamino group, and 15,800 calories for the $\alpha$-amino group. The isoelectric point of canavanine and canaline were 7.93 and 6.46, respectively.

It is shown that one of the basic groups of canavanine is far weaker than the guanidine group of arginine and a little stronger than the imidazole group of histidine. The basic group of canaline other than the $\alpha$-amino group is so weak that it is a little difficult to distinguish it from the $\alpha$-amino group.

(2) The equilibrium constant of the reaction which takes place between certain amino acids and formaldehyde has been calculated. The results show that one molecule of amino acid combines with one molecule of formaldehyde from pH 8.0 to 10.0. The values for glycine, alanine, and proline are $465 \pm 13$, $50.1 \pm 4.9$, and $67.3 \pm 3.4$, respectively.

The mechanism of the reaction in question is discussed. The reaction product most probably may be a molecular compound and not a methylene compound.

(3) The solubility of *l*-proline and *l*-hydroxyproline have been determined with the micro technique which has newly been developed. The solubility-temperature relationships, within the temperature limits studied, can be shown by the following equation:

| | |
|---|---|
| *l*-Proline | $\log S = 3.1050 + 0.4206 \times 10^{-2} t$ |
| *l*-Hydroxyproline | $\log S = 2.4603 + 0.3891 \times 10^{-2} t$ |

The heats of solution of *l*-proline and *l*-hydroxyproline were calculated and found to be 1340 and 1506 calories, respectively.

The apparent molal volume of *l*-hydroxyproline was calculated from the density determinations of the saturated solutions and found to be $85.62 \pm 0.12$ cc. The reported value is in close agreement with that calculated from the empirical atomic volumes given by TRAUBE.

(4) The adsorption by charcoal of each component of several diamino acid-picrates conforms to FREUNDLICH'S adsorption isotherm. The constants of the equation for each picrate have been calculated. The monopicrate of arginine or lysine is adsorbed in molecular proportion of its components. In the case of histidine dipicrate, the histidine cation is adsorbed in an amount less than half of the picrate anion adsorbed.

The probable mechanism of adsorption of these picrates by charcoal has been discussed. The facts which have been obtained in the present investigation can be explained by assuming that the combination of diamino acid picrates with charcoal does not take place at the amino acid cation but at the picrate anion.

*The second part of the dissertation may be summarized under the following conclusions.*

(5) The sources of the error in MAY and ROSE'S method have been examined and a modification for it has been devised. The incubation of the reaction mixture at 30° has been found to be optimum for the development as well as the stability of the color formed by the reaction between tryptophane and the EHRLICH reagent. $6 \sim 7$ days of incubation are necessary in order to reach the maximum color intensity.

It has been indicated that the uncertainty as to the tryptophane content of casein makes this protein of questionable value as a standard. Hence, the blue color given by a standard phosphate solution prepared essentially according to LOHMANN and JENDRASSIK'S modification of FISKE and SUBBAROW'S method has been adopted as a color standard.

(6) Methionine in crystalline form was isolated from the muscle protein of sardine and whale, the protein of silk-worm pupa, the protein of soy bean, and casein. The yield based on dry and ash-free protein was as follows: Muscle protein of sardine, 0.52%; muscle protein of whale, 0.37%; protein of silk-worm pupa,

0.43 %; protein of soy bean, 0.08 %; casein, 0.41 %. Contrary to the results of ODAKÉ, methionine was isolated with certainty, though in small quantity, from the protein of soy bean. Although only a few kinds of protein were investigated in the present study, it might be presumed that methionine would be generally widespread in proteins, especially in those of animal origin.

(7) The cystine contents of several proteins have been determined by OKUDA's iodine method. The methionine contents have been calculated from the values for the total sulphur and cystine-sulphur, based on the assumption that the total sulphur in any protein consists exclusively of methionine-sulphur and cystine-sulphur. It has been shown that the cystine content of the protein of soy bean is higher than that of the protein of sardine, whale, and silk-worm pupa, whereas the content of methionine is quite contrary to that of cystine in the protein of soy bean. Regarding the sulphur containing amino acids as a whole, only a little difference is found between the protein of soy bean and any of the others.

(8) The proteins of sardine, silk-worm pupa, and soy bean were hydrolyzed at 38° by the Kahlbaum's trypsin completely activated by enterokinase, and both the amino-N and the ammonia-N liberated during the digestion were determined after 2, 24, 73, and 163 hours, and the velocities of liberation of these two forms of nitrogen have been compared.

The reactions of liberation of amino-N and ammonia-N were not conformable to the monomolecular reaction. In the initial stage of these digestion, the velocity constant treated as a monomolecular reaction showed markedly greater values and as the digestion progressed their values became gradually smaller. The quantity of amino-N liberated, expressed as per cent, both of total nitrogen and of total amino-N, was greatest in the protein of sardine, and it was far less in the proteins of soy bean and silk-worm pupa. The quantity of the ammonia-N liberated, expressed as per cent of total nitrogen of each of the proteins, was greatest in the protein of soy bean, and there was hardly any difference between that of silk-worm pupa and sardine. But if it is expressed as per cent of the amide-N contained in the protein, it is practically the same in the proteins of soy bean and of sardine, while in the protein of silk-worm pupa it was much smaller than in those of the former two.

(9) A comparative study has been made of KOSSEL's method and SASAKI's method for determining diamino acids. Judging from the yields of derivatives of

diamino acids and their analytical value for nitrogen, KOSSEL's method seems to be superior to SASAKI's method. The isolated amounts of diamino acids by these two methods from 100 g of the protein of sardine were as follows: 0.95 g histidine, 3.85 g arginine, and 5.25 g lysine by KOSSEL's method; 0.55 g histidine, 2.51 g arginine. and 2.60 g lysine by SASAKI's method.

(10) The effects of the various substances on the determination of amino acid by the VAN SLYKE method have been investigated, and found to be such that the contamination of carbohydrate interferes considerably with the values for humin- and arginine-N, and that the presence of fat influences the results to a negligibly small extent. The method, accordingly, can not be applied directly to the analysis of protein of foods or feedstuffs. The method for isolating the protein part of the sample has been examined, using soy bean, sardine, and silk-worm pupa. It has been found that the protein of these materials can be coagulated to the extent of 91 ~ 95 per cent of the total protein by boiling the materials with water acidified with acetic acid, thus leaving most of the carbohydrate except cellulose in the solution.

The total coagulable proteins of soy bean, sardine, and silk-worm pupa have been analyzed by the VAN SLYKE method. It has been shown that the proteins of sardine and silk-worm pupa give a similar distribution of various forms of nitrogen, but the protein of soy bean, compared with these two proteins, gives higher value for arginine-N and lower values for lysine- end histidine-N.

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